2,3-Butanediol Production with GRAS Microorganisms – Screening, Cultivation, Optimization and Scale-Up –

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To my grandparents

The important thing is to never stop questioning.

− Albert Einstein −

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1 Introduction and aims

During the last few years, in the face of the inevitable end of crude oil reserves and the rapid increase of petroleum prices, bio-based processes for the production of bulk-chemicals have become increasingly important (Hatti-Kaul et al. 2007; Ji et al. 2011). The use of low-cost renewable feedstock is essential for the development of efficient industrial fermentation processes. A promising substrate is excess biomass, such as food industry residues, biodiesel by-produced glycerol or wood-based lignocellulosic biomass (Willke and Vorlop 2004; Celińska and Grajek 2009; Zeng and Sabra 2011).

2,3-Butanediol (hereafter referred to as 2,3-BD) is an important bulk-chemical with a large range of potential industrial applications. By developing an efficient bio-based process for the microbial production of 2,3-BD from renewable resources, fossil fuel supplies can be preserved and environmental benefits can be obtained (Ji et al. 2011).

1.1 2,3-Butanediol

1.1.1 Physico-chemical properties

2,3-BD, also known as 2,3-butylene glycol, dimethylene glycol and 2,3-dihydroxybutane, is a chiral bivalent alcohol. The IUPAC name is butane-2,3-diol, the molecular formula $C_4H_{10}O_2$ and the molecular weight 90.212 g/mol. 2,3-BD is colorless, odorless, strongly hygroscopic, soluble in water and with a sweet taste (Voloch et al. 1985, Gräfje et al. 2000).

Due to the presence of two chiral centers, there are three possible stereoisomeric forms for 2,3-BD (Fig. 1-1). The optically inactive isomer *(R,S)*-2,3-BD is also known as *meso*-form; the two optically active forms are *(2R,3R)*-2,3-BD [or *D-(-)*-; levo-isomer] and (*2S,3S*)-2,3-BD [or *L-(+)*-; dextro-isomer] (Gräfje et al. 2000; Celińska and Grajek 2009; Ji et al. 2011).

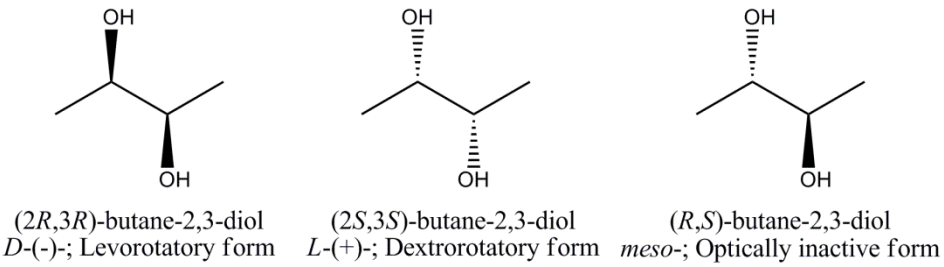


Figure 1-1 Stereoisomers of 2,3-BD (modified after Celińska and Grajek 2009; Ji et al. 2011)

Depending on the stereoisomeric form, at room temperature 2,3-BD can appear as an oily liquid or crystalline solid. The boiling points of the three isomers range from 177 to 182°C, while the melting points vary between 19 and 36.5°C. The racemate [*(2R,3R)*- and *(2S,3S)*-

2,3-BD] has a boiling point of 176.7°C and a melting point of 7.6°C (Voloch et al. 1985; Gräfje et al. 2000).

1.1.2 Importance of 2,3-BD

2,3-BD has a wide range of potential industrial applications. Among others, it can be used as carrier for pharmaceuticals as well as for the production of moistening and softening agents, perfumes, fumigants, insecticides, explosives, plasticizers and printing inks (Magee and Kosaric 1987; Garg and Jain 1995; Ji et al. 2011). 2,3-BD has a heating value of 27,198 kJ/kg, which makes it a promising fuel additive comparable to other liquid fuels like methanol (22,081 kJ/kg) and ethanol (29,055 kJ/kg) (Flickinger 1980). The potential application of 2,3-BD as cryoprotectant was tested during the nonfreezing, subzero preservation of rat liver (Soltys et al. 2001).

Dehydration of 2,3-BD leads to methyl ethyl ketone (MEK), an important solvent fuel additive with a combustion heat higher than ethanol (Magee and Kosaric 1987). MEK can also be used as solvent for resins and lacquers (Villet 1981). Furthermore, 2,3-BD can be converted to 1,3-butadiene (1,3-BD), which is the building block of synthetic rubber (van Haveren et al. 2007; Syu 2001; Celińska and Grajek 2009). Diacetyl, a high-value food additive, is formed by catalytic dehydrogenation of 2,3-BD. It can be employed as flavoring agent due to its buttery taste and is also an important bacteriostatic food additive (Bartowsky and Henschke 2004; Macciola et al. 2008). By esterification of 2,3-BD precursors of polyurethane foams with antiseptic properties and application in cosmetic products, lotions, drugs, antiperspirants and ointments can be obtained (Garg and Jain 1995; Syu 2001). Polyurethane-melamides (PUMA's) are synthesized by esterification of 2,3-BD with maleic acid and can be used in cardiovascular applications (Petrini et al. 1999; Celińska and Grajek 2009).

1.1.3 Chemical and microbial production

The chemical synthesis of 2,3-BD is based on the C_4 hydrocarbon fraction obtained from crack gases after butadiene and isobutene have been removed. This fraction contains up to 77% butenes, while the residual 23% correspond to a mixture of butane and isobutane. The C_4 hydrocarbon fraction is subjected to a chlorination step using a chlorine water solution. Afterwards, the chlorohydrins are cyclized with sodium hydroxide, yielding a mixture of 55% *trans*-2,3-butene oxide, 30% *cis*-2,3-butene oxide and 15% 1,2-butene oxide. After hydrolysis at 160-220°C and 50 bar, a mixture of butanediols results, which are easily separated by vacuum fractionation. An excess of water during hydrolysis ensures that no polyethers are produced. During fractionation, *trans*-2,3-butene oxide is converted to *meso*-2,3-BD, while *cis*-2,3-butane oxide leads to the racemic mixture of *[2R,3R]*- and *[2S,3S]*-2,3-BD (Gräfje et al. 2000).

Compared to the chemical synthesis, the biotechnological route for 2,3-BD production using microorganisms is cheaper. Due to the ecological advantages of a sustainable production by the use of renewable raw materials, the microbial synthesis represents a promising route for commercial 2,3-BD production (Zeng and Sabra 2011).

Microbial 2,3-BD synthesis dates back to 1906, when Harden and Walpole first reported 2,3-BD production using *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*) (Garg and Jain 1995; Celińska and Grajek 2009). The first proposal for an industrial-scale production of 2,3-BD was made by Fulmer et al. (1933) and research on 2,3-BD fermentation began to thrive during World War II due to a strong demand for 1,3-butadiene (Voloch et al. 1985; Ji et al. 2011). Since then, numerous efforts have been made in searching for better performing microbial strains and improving fermentation processes.

1.2 Microbial 2,3-BD producers

Many microbial strains are able to synthesize 2,3-BD from pyruvate. Species belonging to the genera *Klebsiella*, *Enterobacter*, *Bacillus* and *Serratia* are considered promising for industrial 2,3-BD production (Maddox 1996). Research has been conducted using the strains *Klebsiella oxytoca* (Jansen et al. 1984; Ji et al. 2009a), *Klebsiella pneumoniae* (formerly *Aerobacter aerogenes*; Yu and Saddler 1982, 1983; Grover et al. 1990), *Enterobacter aerogenes* (Perego et al. 2000; Converti et al. 2003) and *Serratia marcescens* (Ui et al. 1983; Zhang et al. 2010a, b). These microorganisms belong to the risk group 2 (pathogenic), which makes them unsuitable for industrial-scale fermentation because of the strict safety regulations and increasing costs of the process (Celińska and Grajek 2009).

On the contrary, using risk group 1 strains, an industrial fermentation process would be cheaper and less complex (Yang et al. 2011). A number of studies describe employing non-pathogenic strains for 2,3-BD production: *Paenibacillus polymyxa* (formerly *Bacillus polymyxa*; de Mas 1988; Nakashimada et al. 1998, 2000; Marwoto et al. 2004), *Bacillus licheniformis* (Nilegaonkar et al., 1992, 1996; Perego et al. 2003), *Bacilllus subtilis* (Liu et al. 2011) and *Bacilllus amyloliquefaciens* (Alam et al. 1990). However, compared to the risk group 2 strains the maximum 2,3-BD concentrations reached with these bacteria are much lower (Ji et al. 2011). Thus 2,3-BD production with non-pathogenic strains must be increased so they can serve as potential producers for 2,3-BD fermentation in large scale.

Different microorganisms are able to synthesize different stereosiomers of 2,3-BD; however, most strains are known to yield a mixture of two isomers. Strains of the genus *Klebsiella* and *Enterobacter* produce *L-(+)*- and *meso*-2,3-BD, members of the genus *Bacillus* generate *D-(-)*- and *meso*-2,3-BD, while *P. polymyxa* yields pure *D-(-)*-2,3-BD (Ji et al. 2011)

1.3 Metabolic pathway

2,3-BD is produced from pyruvate via the mixed acid fermentation pathway in three steps. The first step is the decarboxylation of pyruvate to α-acetolactate in the presence of the α-acetolactate synthase. α-Acetolactate can be further converted to acetoin by α-acetolactate decarboxylase. In the last step, the reversible reduction of acetoin to 2,3-BD is catalyzed by acetoin reductase (also known as butanediol dehydrogenase; Maddox 1996; Celińska and Grajek 2009; Ji et al. 2011). Fig. 1-2 illustrates the mixed acid fermentation pathway for 2,3-BD and by-product synthesis. The main by-products are glycerol, ethanol, acetate, succinate, lactate and formate. Furthermore, acetate was shown to induce the three enzymes involved in pyruvate conversion to 2,3-BD (Stormer 1968; Bryn et al. 1973; Nakashimada et al. 2000).

For the production of 2,3-BD, the monosaccharides contained in the sugar source (C-source) must first be converted to pyruvate. In the case of hexoses (e.g. glucose) pyruvate is formed via the Embden-Meyerhof-Parnas pathway (EMP pathway or gycolysis), while the degradation of pentoses (e.g. xylose) requires a combination of the pentose phosphate and the EMP pathways (Jansen and Tsao 1983; Ji et al. 2011).

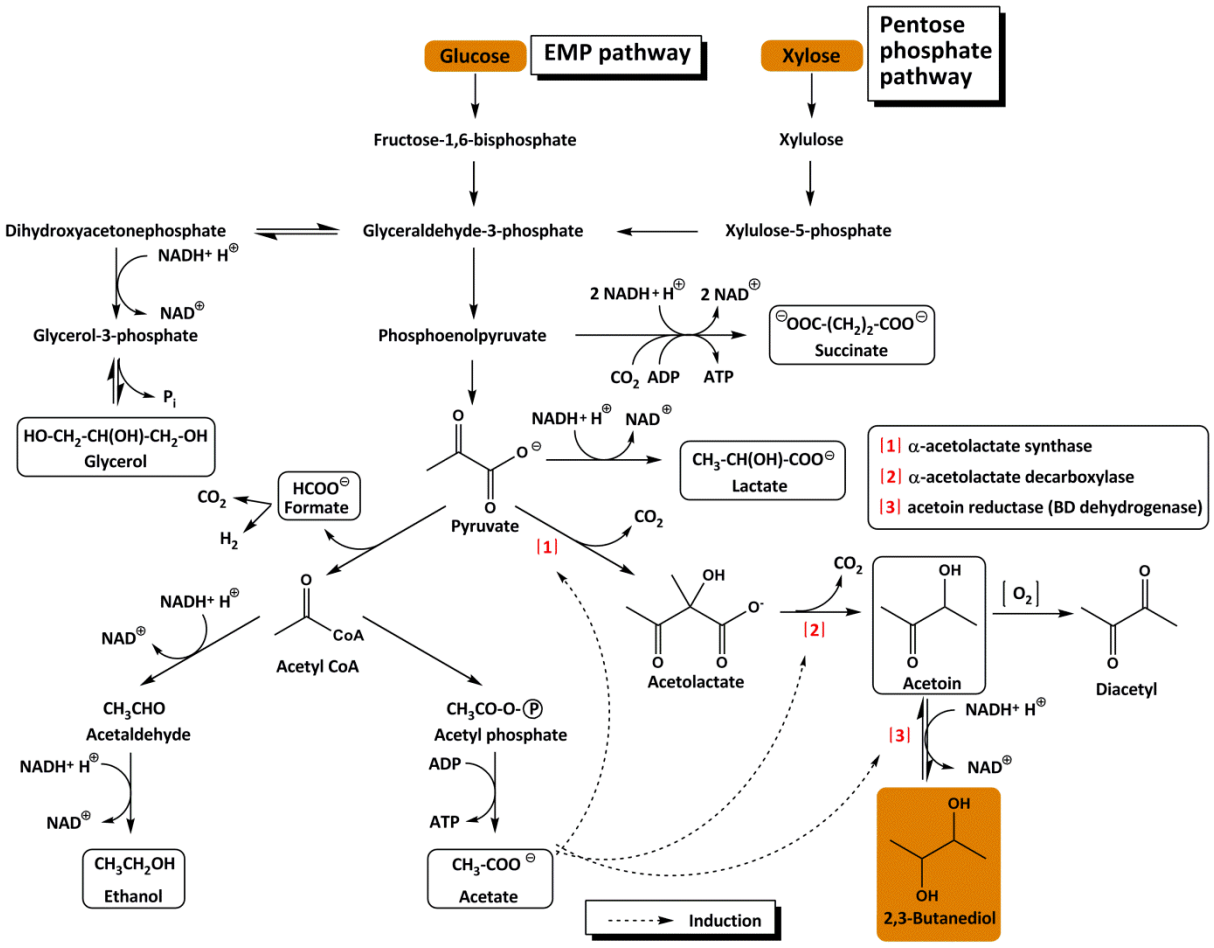


Figure 1-2 Mixed acid fermentation pathway (after Maddox 1996; Henriksen and Nilsson 2001)

1.4 Physiological function

Up to now, the metabolic function of 2,3-BD production has not been well clarified. However, there are three significant physiological roles for 2,3-BD biosynthesis: preventing intracellular acidification, regulating the NADH/NAD^+ ratio and storing carbon and energy (Celińska and Grajek 2009; Ji et al. 2011).

The role of 2,3-BD production in preventing intracellular acidification is manifested by changes in the metabolism from acid production to the formation of neutral compounds like 2,3-BD (Blomqvist et al. 1993). The accumulation of acidic products in the medium leads to a decrease in extracellular pH; the resulting transmembrane pH gradient causes intracellular acid accumulation, which induces 2,3-BD production (Booth 1985; van Houdt et al. 2007).

Due to the reversible reaction between acetoin and 2,3-BD, the pathway has been considered to participate in the maintenance of the intracellular NADH/NAD^+ balance (Blomqvist et al. 1993; Maddox 1996). During glycolysis a net conversion of NAD^+ to NADH occurs. NAD^+ can be regenerated by the reduction of acetoin to 2,3-BD. After glucose is exhausted and NADH production stops, the reverse reaction occurs and extracellular 2,3-butanediol acts as a reservoir of reducing equivalents (Johansen et al. 1975; Magee and Kosaric 1987; Maddox 1996).

2,3-BD biosynthesis is also a bacterial carbon and energy storage strategy. During the stationary phase, when other carbon and energy sources have been depleted, microorganisms are able to degrade, i.e. reutilize 2,3-BD (Xiao and Xu 2007).

1.5 Substrates

Microorganisms are able to ferment a broad variety of substrates to 2,3-BD, including pure sugars, non-cellulosic and lignocellulosic substrates (Ji et al. 2011). For the development of an economical fermentation process, the use of inexpensive carbohydrate raw materials is essential, since the major cost of the process and the cost of the final product are given by the substrate cost (Voloch et al. 1985; Willke and Vorlop 2004).

1.5.1 Pure sugars

The most common substrates employed for 2,3-BD production are carbohydrates. *K. oxytoca* and *P. polymyxa* are able to grow on a variety of hexoses and pentoses, including glucose, mannose, galactose, xylose, arabinose, as well as on cellobiose, lactose and sucrose (de Mas et al. 1988; Champulvier et al. 1989; Marwoto et al. 2004; Ji et al. 2009b). *P. polymyxa* can additionally ferment polymeric substances like xylan, inulin and starch (Maddox 1996).

Wang et al. (2010) showed that there is a preference concerning sugar consumption for *K. pneumoniae*: glucose is preferred over arabinose, which is favored over xylose.

Furthermore, research studies employing *K. pneumoniae* showed high 2,3-BD concentrations on medium containing glycerol as carbon source (Petrov and Petrova 2009, 2010).

1.5.2 Non-cellulosic substrates

Among the potential low cost non-cellulosic substrates for 2,3-BD production are food industry residues like starch hydrolysates derived from corn transformation, molasses from sugar beet extraction and whey from cheese manufacture, which resulted in high values for 2,3-BD yield and volumetric productivity (Perego et al. 2000, 2003). The use of whey permeate was investigated in a series of studies, but the results were low when free cells of *K. pneumoniae* were employed for cultivation (Barrett et al. 1983; Lee and Maddox 1984). With immobilized cells of *K. pneumoniae* however, higher productivities were achieved (Lee and Maddox 1986). The use of molasses from sugar cane juice was also studied and 2,3-BD concentrations above 100 g/L were obtained with *K. oxytoca* (Afschar et al. 1991, 1993).

Other promising alternative non-cellulosic substrates are hexose rich plants like Jerusalem artichoke and sugar cane. 2,3-BD production from Jerusalem artichoke tubers using *K. pneumoniae* was successfully investigated by Sun et al (2009). Li et al. (2010) employed both the Jerusalem artichoke tuber and stalk as substrate. Gao et al. (2010) studied 2,3-BD production with *P. polymyxa* and raw inulin extract from Jerusalem artichoke. Cultivations using sugar cane bagasse and *K. pneumoniae* were conducted by Song et al. (2012).

A further potential low cost non-cellulosic substrate is biodiesel by-produced glycerol. Metsoviti et al. (2012) screened for potential microbial strains for 2,3-BD production from raw glycerol; *E. aerogenes* and *K. oxytoca* isolates were found suitable for this process.

1.5.3 Lignocellulosic substrates

Lignocellulosic materials are composed of sugars polymerized to cellulose and hemicellulose, which are liberated during hydrolysis (Palmqvist and Hahn-Hägerdal 2000).

An example of a low cost lignocellulosic substrate and widely available agricultural residue is corn cob (Ji et al. 2011). Cao et al. (1997) investigated 2,3-BD production from corn cob by *K. oxytoca* in a simultaneous saccharification and fermentation (SSF) process. Cheng et al. (2010) employed detoxified corncob acid hydrolysate and *K. oxytoca*, while Wang et al. (2010) used corncob molasses, a waste by-product in xylitol production, and *K. pneumoniae*.

Another type of lignocellulosic substrate for 2,3-BD production are wood hydrolysates. *K. pneumoniae* is able to produce 2,3-BD from both acid and enzymatically hydrolyzed wood

hemicellulose (Yu et al. 1982, 1985). Since most microorganisms are not able to degrade lignocellulosic biomass, a pretreatment is required. Fig. 1-3 shows a scheme for the steam-refining pretreatment of wood. However, some strains like *P. polymyxa* produce extracellular xylanases, which makes the utilization of most parts of the hemicellulose fraction of lignocellulosic materials possible (Hespell 1996). Another possibility is the co-fermentation of *K. pneumoniae* and *Trichoderma harzianum*, using the cellulolytic and xylanolytic enzymes of the fungus for hydrolyzing the lignocellulosic substrate (Yu et al. 1985).

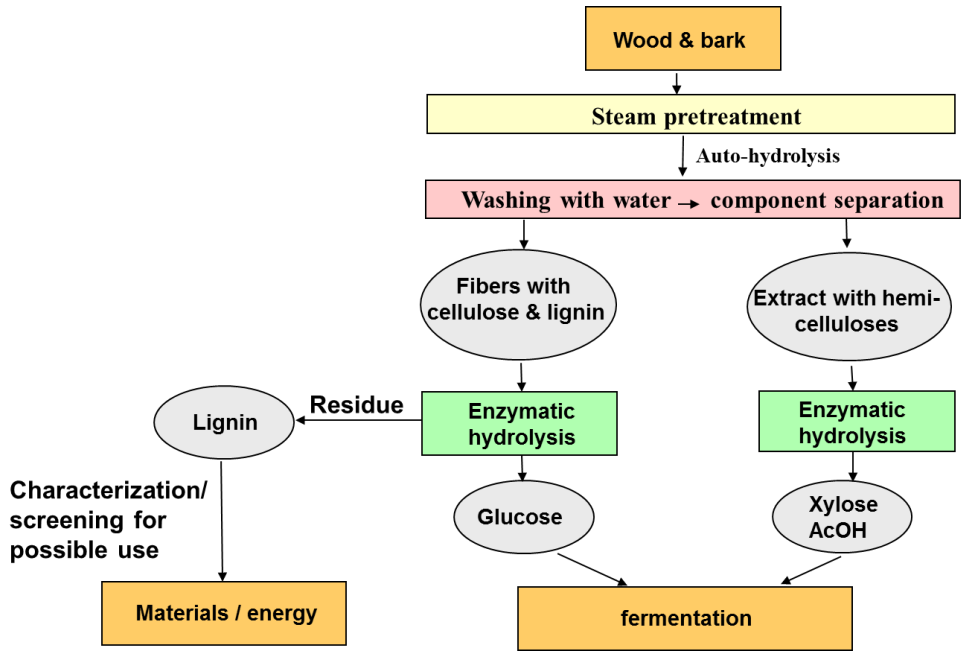


Figure 1-3 Scheme for steam-refining pretreatment of poplar wood (Schütt et al. 2009)

The main components of wood biomass are cellulose, hemicellulose and lignin. Cellulose is a high molecular weight linear polymer consisting of β (1→4) linked D-glucose units. Hemicelluloses are branched polysaccharides containing D-xylose, L-arabinose, D-mannose, D-glucose, D-galactose and uronic acids (Palmqvist and Hahn-Hägerdal 2000; Saha 2003).

During hydrolysis of lignocellulosic materials, a variety of compounds which may inhibit microbial growth are formed (Fig. 1-4). The potential inhibitory compounds are divided into three groups: furans (furfural, 5-hydroxymethyl furfural), phenolic compounds (vanillin, syringaaldehyde, 4-hydroxy benzoic acid) and organic acids (acetic acid, formic acid) (Palmqvist and Hahn-Hägerdal 2000). Hemicellulose hydrolysis leads to sugars and acetic acid, while further degradation of xylose results in furfural production. 5-HMF is formed by cellulose hydrolysis. Formic acid is obtained when furfural and 5-HMF are further degraded (Dunlop 1948; Ulbricht et al. 1984). Partial breakdown of lignin leads to the production of phenolic compounds (Bardet et al. 1985).

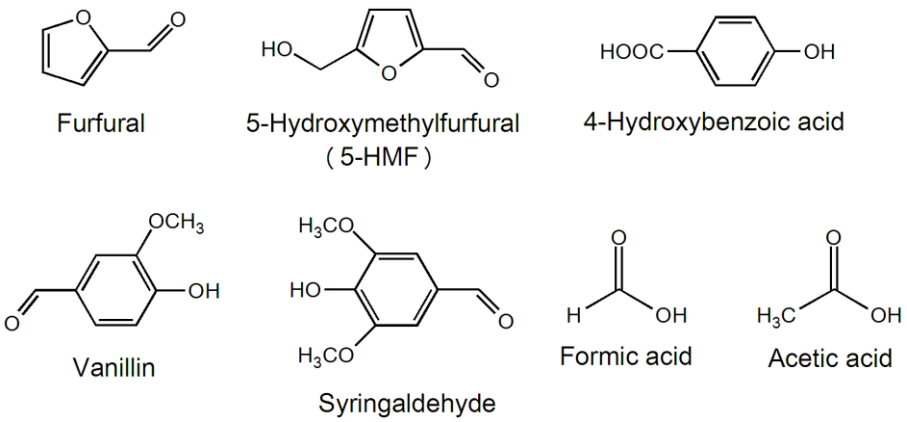


Figure 1-4 Potential inhibitory compounds present in wood hydrolysates

1.6 Factors affecting 2,3-BD production

A series of parameters (e.g. medium components, temperature, pH, aeration) has been shown to affect 2,3-BD production. In this chapter, the different factors are introduced.

1.6.1 Medium composition

Apart from the essential nutrients required for bacterial growth and maintenance, for an efficient 2,3-BD synthesis some additives like vitamins and trace elements have to be supplemented (Ji et al. 2011).

Yeast extract (YE), urea, ammonium salts and trace elements are important for protein synthesis, which play a role in the biomass and enzyme generation (Ji et al. 2011). Laube et al. (1984b) studied the effect of yeast extract on 2,3-BD production from glucose by *P. polymyxa* and a level of 1.5% (w/v) was found optimal. However, results were similar with a level of 0.5% YE, when the phosphate concentration was increased and iron and manganese were added to the culture medium (Laube et al. 1984b). Due to the cost of complex nutrients like YE, attempts have been made in order to find suitable compounds for YE replacement. Urea and ammonium sulfate were identified as potential candidates (Sivakumar et al. 1995).

For *K. pneumoniae* a culture medium containing glucose and ammonium phosphate was constructed based on response surface experiments, leading to 2,3-BD concentrations above 50 g/L in batch and 90 g/L in fed-batch cultivations (Qin et al. 2006).

Nakashimada et al. (2000) reported an enhanced 2,3-BD production on medium with acetate, succinate, pyruvate or propionate supplementation using *P. polymyxa*. Acetate was proven to be the best inducer, leading to the highest yield and product concentration. The addition of malate, formate, lactate, butyrate and valerate showed no effect on 2,3-BD production (Celińska and Grajek 2009).

1.6.2 Initial sugar concentration

For cultivation experiments on medium containing pure sugars and no inhibitory compounds, initial sugar concentrations up to 200 g/L are possible and 2,3-BD yield is still increasing (Jansen et al. 1984).

The specific growth rate of *K. oxytoca* was shown to decrease with increasing initial xylose concentration due to the decreasing water activity. However, maximum values for 2,3-BD productivity were obtained at initial sugar concentrations around 100 g/L xylose (Jansen et al. 1984). Similar results were reported by Sablayrolles and Goma (1984) for *K. oxytoca* growing on glucose.

Research studies carried out with *P. polymyxa* showed that at glucose concentration higher than 150 g/L growth inhibition phenomena occurred (de Mas et al. 1988). During cultivations performed with *B. licheniformis*, the best results regarding volumetric productivity were reached with an initial glucose concentration of 30 g/L (Perego et al. 2003).

Regarding cultivation experiments performed using renewable resources as sugar source, by increasing the initial sugar level an increase in the concentration of potential inhibitory compounds occurs (Maddox 1996). The most common initial sugar concentrations are in the range of 5-10%, so that the amounts of inhibitors are not too high (Garg and Jain 1995).

1.6.3 Temperature

For most 2,3-BD producing bacteria, the optimum temperature lies in the range of 30-37°C (Maddox 1996). Since 2,3-BD synthesis is a growth-associated phenomenon, the optimum temperature for product formation must be similar to the optimum temperature for maximum biomass yield (Garg and Jain 1995).

Barrett et al. (1983) reported that a decrease in 2,3-BD production by 66% was obtained when the temperature for the cultivation of *K. pneumoniae* was increased from 33 to 37°C. In the case of *E. aerogenes*, temperatures in the range from 23 to 46°C were tested and an optimum value of 39°C was found (Converti et al. 2003). A temperature of 30°C was determined as optimal for batch and fed-batch cultivation using *P. polymyxa* (Hespell 1996; Nakashimada et al. 2000).

The optimum temperature depends on the strain and substrate employed for cultivation. Therefore the optimal value should be determined individually for each case (Celińska and Grajek 2009).

1.6.4 pH value

Both the biomass composition and the nature of bacterial metabolism are influenced by the pH of a cultivation medium (Garg and Jain 1995).

In general, starting pH values above 6.3-6.5 favor the production of organic acids and consequently 2,3-BD yield decreases. Organic acid production during cultivation results in culture acidification and increasing levels of toxic undissociated forms of acids. As a consequence, growth and product turnover slowly stop, until the products finally inactivate the culture (Biebl et al. 1998). On the other hand, pH values below 6.3 lead to a more than 10-fold reduction of organic acid synthesis, corresponding to a 3-7-fold increase in 2,3-BD formation (Garg and Jain 1995).

Some microbial strains have developed a defense mechanism against acidification by switching the metabolism from acid production to the synthesis of less toxic compounds like alcohols and 2,3-BD (van Houdt et al. 2007).

Like in the case of the temperature, the optimum value for the pH depends on the microorganism and substrate employed (Celińska and Grajek 2009). For most substrates (including wood hydrolysates), the optimum pH value is in the range of 6.0-6.2 (Grover et al. 1990). Maximum 2,3-BD production from glucose with *B. licheniformis* was obtained at a pH of 6.0 (Raspoet et al. 1991). On medium containing xylose, the highest product formation occurred at a pH of 5.0-5.2 (Tsao 1978). For 2,3-BD production an optimum pH of 5-6 was found using *K. oxytoca* (Voloch et al. 1985), while a pH of 6 was chosen for *E. aerogenes* (Converti et al. 2003). Using *P. polymxya* a pH in the range of 6.3-6.8 was found best for 2,3-BD formation (Nakashimada et al. 2000).

1.6.5 Aeration

The most important parameter for 2,3-BD production is considered to be the oxygen supply. 2,3-BD production is considered a product of anaerobic fermentation; however, aeration was shown to enhance its synthesis (Maddox 1996). Furthermore, Kosaric et al. (1992) reported that a too high oxygen supply prevents 2,3-BD production by rapid and irreversible inactivation of the α-acetolactate synthase. Additionally, Jansen et al. (1984) noted that high oxygen supply favored the production of cell mass at the expense of 2,3-BD synthesis.

Facultative anaerobic microorganisms like *Klebsiella* sp. and *P. polymyxa* are able to obtain energy by two different pathways: respiration and fermentation. Both pathways are active simultaneously during limited oxygen supply. By reducing the oxygen supply, 2,3-BD yield can be increased; cell mass decreases and fermentation is preferred over respiration.

By increasing the oxygen supply, the amount of cell mass is enhanced and respiration is the preferred metabolism, leading to CO_2 formation. The spectrum of product formation is influenced by the relative oxygen availability. The effect of aeration on *P. polymyxa* is shown in Fig. 1-5. (Celińska and Grajek 2009).

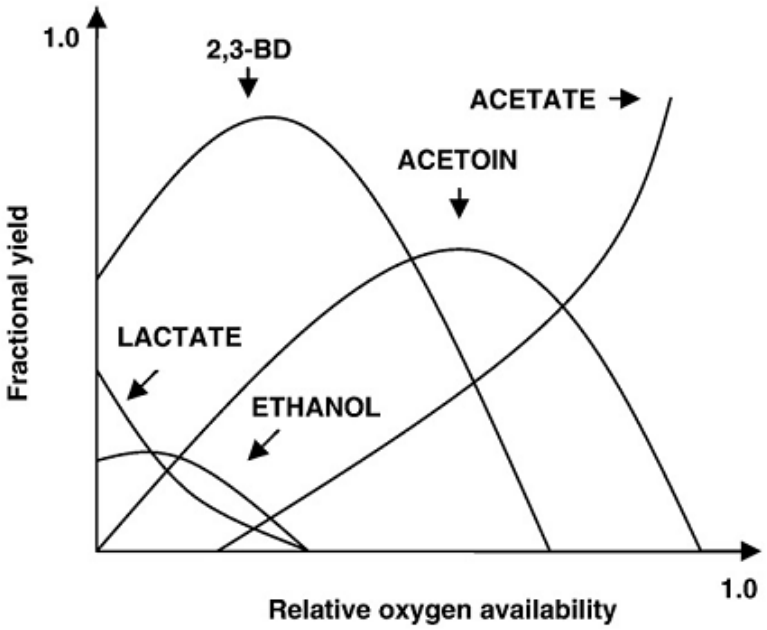


Figure 1-5 The effect of relative oxygen availability on fractional product yields by *P. polymyxa* (the figure is an approximation; after de Mas et al. 1988; Maddox 1996)

Acetoin reduction to 2,3-BD is reversible. Moes et al. (1985) reported an increased acetoin formation at high oxygen supply rates. Dissolved oxygen (DO) levels above 100 ppb led to acetoin excretion, while 2,3-BD production was induced at DO levels below 100 ppb.

1.6.6 Agitation

Aeration is commonly associated with agitation. Stirring is important in fermentation processes because it ensures an efficient fermentation by constantly exposing new substrate to the microorganisms and distributing the fermentation products in the culture broth (Celińska and Grajek 2009).

During experiments using *K. pneumoniae* and *E. aerogenes* a stirring rate of 220 rpm was found optimal, leading up to a 5-fold and 15-fold enhancement of 2,3-BD production from glucose and lactose, respectively (Barrett et al. 1983).

However, too high agitation values correspond to high aeration rates and lead to a decrease in 2,3-BD production. Cultures of *P. polymyxa* shaken at 125 rpm showed higher amounts of 2,3-BD compared to those shaken at 300 rpm (Laube et al. 1984a).

1.7 Scale-up

Regarding bioreactor cultivations, research has been conducted on different fermentation conditions in order to optimize 2,3-BD production.

1.7.1 Oxygen supply control

As already mentioned in chapter 1.6.5, the oxygen supply is a critical factor affecting 2,3-BD production, yield and productivity.

In the research study of Beronio and Tsao (1993) the influence of the oxygen transfer rate (OTR) on 2,3-BD formation with *K. oxytoca* was investigated. The OTR was employed to maintain growth rate and specific oxygen uptake rate (OUR). In OTR-controlled fermentation experiments, 2,3-BD productivity increased by 18%.

Furthermore, the effect of volumetric oxygen transfer coefficient (k_La) was also studied. For 2,3-BD production with *P. polymyxa*, a programmed variation of k_La process was employed. The k_La was set at three different levels: 40 h^{-1} (0-19 h); 21 h^{-1} (19-41 h) and 8 h^{-1} (41-55 h). Using this method, 2,3-BD production reached levels of 44 g/L with a productivity of 0.79 g/(L*h) (Fages et al. 1986).

Using the continuous cultivation mode, Zeng et al. (1990) determined OUR rates and observed that the levels varied according to the dilution rate (D). Different OUR values were obtained by changing the impeller speed using a constant aeration rate. At all dilution rates studied, cell mass increased gradually with increasing OUR. At each D an optimal OUR giving highest volumetric productivity for the sum of butanediol and acetoin was found.

Zeng et al. (1994) attempted a control strategy for 2,3-BD production using the respiratory quotient (RQ). An RQ between 4.0 and 4.5 was found optimal for 2,3-BD production. These results were in accordance with the optimal RQ value of 4.0, obtained stoichiometrically. Using an RQ control algorithm for oxygen supply, production concentrations above 100 g/L (2,3-BD plus acetoin) were obtained during fed-batch cultivations with *E. aerogenes*. Additionally, the RQ was successfully used as a control parameter for the scale-up of 2,3-BD production from laboratory to pilot plant scale reactors.

A suitable control strategy was constructed by Zhang et al. (2010b) by combining an RQ control with a constant residual sucrose concentration during fed-batch cultivation with *S. marcescens*. Based on stoichiometric calculations, the RQ value was set to 1.0-1.5 for cell growth and 1.8-2.0 for 2,3-BD production, respectively.

Since an OTR, k_La, OUR and RQ control is not easy to implement, Ji et al. (2009a) tested a two-stage agitation speed control strategy using *K. oxytoca* and achieved promising results.

1.7.2 Reactor operation mode

A series of reactor operation modes were tested for optimum 2,3-BD production, including batch, fed-batch, continuous culture, cell recycle and immobilized cell systems (Ji et al. 2011).

A high product concentration is important for an efficient fermentation process, but difficulties arise due to 2,3-BD recovery, which is usually achieved by steam stripping, pervaporation or solvent extraction (Xiu and Zeng 2008). In order for 2,3-BD recovery to be feasible, product concentrations above 80 g/L are required (Magee and Kosaric 1987).

Using the fed-batch cultivation mode, high final 2,3-BD concentration could be obtained, simultaneously reducing the effect of initial substrate inhibition (Ramachandran et al. 1990). Yu and Saddler (1983) developed a double-fed batch technique for *K. pneumoniae* and increased the sugar content by 20 g/L daily, leading to over 100 g/L 2,3-BD from glucose and over 80 g/L from xylose. Qin et al. (2006) used the fed-batch cultivation mode and sterilized glucose in solid form for the feedings, which resulted in 2,3-BD concentrations above 90 g/L after 50 h of cultivation with *K. pneumoniae*.

Using a continuous cultivation mode, 2,3-BD productivity was increased, due to the fact that the reactor was operated at steady state near maximum reaction rate. Successful enhancement of 2,3-BD productivity was reported by Lee and Maddox (1986) and Zeng et al. (1990 a, b).

Ramachandran and Goma (1988) employed a continuous cell recycle system for efficient 2,3-BD production with *K. pneumoniae*. Higher 2,3-BD productivities were obtained compared to batch and continuous fermentation systems. A portion of the ultrafiltrate was removed from the system to maintain the dilution rate, while another portion was recycled back to the reactor together with the cells by the use of an ultrafiltration unit. Furthermore, a cell recycle system with a microfiltration module was developed by Zeng et al. (1991). The system was employed during cultivation with *E. aerogenes*, which resulted in a three-fold increase of 2,3-BD productivity, up to 14.6 g/(L*h).

Besides using different cultivation modes, further research studies employed immobilized cells for 2,3-BD production. *K. pneumoniae* cells immobilized in calcium alginate were added to a packed column reactor and efficient 2,3-BD production from whey permeate was achieved by Lee and Maddox (1986).

1.7.3 Process control and automation

During cultivation experiments with *B. subtilis*, Moes et al. (1985) showed that by changing the level of dissolved oxygen (DO), the reversible conversion of acetoin to 2,3-BD can be enabled. A DO control system was implemented in order to maintain the DO at a constant level and monitor the culture response to modifications in stirring speed during cultivation.

Zeng and Deckwer (1992) introduced a new parameter for 2,3-BD production with *E. aerogenes*. The TCA cycle was used to quantitatively account for the involvement of fermentation pathways and for the utilization of oxygen under microaerobic conditions. Exhaust gas analysis and monitoring of metabolite concentrations were employed for the estimation of the parameter, which was shown to be helpful as reactor design criterion for microaerobic cultivation.

For an efficient and successful online monitoring and process control system, fast and reliable measuring devices are required. An example of such a device employed for online measurement of fermentation products was designed by Hayward et al. (1991). The device comprises the membrane introduction mass spectrometry (MIMS) method coupled with a flow injection analysis (FIA) sampling system. The MIMS method enables a selective permeation of the solutes, while the FIA sampling system helps monitoring the production of 2,3-BD, acetoin, ethanol and acetic acid.

A further automation method was proposed by Syu and Hou (1997) employing neural network computation for the study of dynamic identification and prediction of a fermentation system for producing 2,3-BD. During cultivation with *K. oxytoca*, 2,3-BD and the fermentation by-products were measured online at the same time by the MIMS method. A back-propagation neural network was then applied in order to help modeling the experimental data obtained by online monitoring (Syu and Tsao 1993). A very good correlation was found between the simulation results of the network and the experimental data. Furthermore, the neural network enabled an accurate prediction of experimental data by interpolation or extrapolation.

1.8 Strain improvement

An improvement of 2,3-BD production can be achieved by targeted or untargeted (random) mutagenesis. In targeted mutation, the genes are directly located and desired modifications are performed. In untargeted mutation, genes are randomly mutated using chemicals or UV radiation, followed by a selection process for picking out the optimized mutant strains.

1.8.1 Random mutagenesis and mutant selection

Random mutagenesis using UV coupled with diethyl sulfate (DES) was carried out for *K. oxytoca* wild type strain ME-303 followed by a modified proton suicide selection strategy (Ji et al. 2008).

The proton suicide method is employed to select strains producing less organic acids. It is based on the redox reactions between bromine in selective media and the protons (H^+) derived from organic acids (Winkelman and Clark 1984). In the presence of excess H^+, bromate (BrO_3^-) and bromide (Br^-) lead to the formation of elementary bromine (Br_2):

$$BrO_3^- + 2Br^- + 2H^+ = Br_2 + BrO_2^- + H_2O \quad \textbf{(Equation 1-1)}$$

The bromite (BrO_2^-) further reacts with H^+ to produce hypobromite (BrO^-):

$$BrO_2^- + 2Br^- + 2H^+ = Br_2 + BrO^- + H_2O \quad \textbf{(Equation 1-2)}$$

The hypobromite is unstable and a disproportionation into bromine and bromate follows:

$$3BrO^- = Br_2 + BrO_3^- \quad \textbf{(Equation 1-3)}$$

Therefore, the overall reaction is:

$$2BrO_3^- + 12Br^- + 12H^+ = 7Br_2 + 6H_2O \quad \textbf{(Equation 1-4)}$$

The proton suicide method involves a lethal synthesis of acid producing mutants. Cells producing excess protons are killed by the reaction of these protons with bromate and bromide, due to the toxic effect of the resulting bromine. Nonacid producing mutant strains survive the selection step (Winkelman and Clark 1984). Among the advantages of the proton suicide selection are the applicability to a wide range of media and microorganisms and the enabling of an easy selection directly from agar plates. However, the method is only relevant for acid-producing microorganisms (Winkelman and Clark 1984).

Results of the aforementioned study of Ji et al. (2008) showed that mutants (ME-UD-3) with defects in acid formation pathways could be successfully isolated; by-product synthesis of lactic acid and acetic acid were strongly diminished by 88% and 92%, respectively.

1.8.2 Targeted genetic modifications

Targeted insertional mutagenesis can be applied in order to eliminate or significantly reduce by-product formation during 2,3-BD synthesis, so that 2,3-BD yield can be enhanced. The principle of this method is to insert one or more bases into normal DNA sequences, thus leading to deficiencies in coding specific enzymes.

During cultivation with the previously mentioned ME-UD-3 mutant of *K. oxytoca*, ethanol was obtained as major by-product (Ji et al. 2008). The gene insertional inactivation technology was employed for this strain by inactivating the *aldA* gene coding for aldehyde dehydrogenase. This way ethanol production was blocked, which resulted in a redirection of the carbon flux towards 2,3-BD formation. During fed-batch cultivation with the ethanol deficient mutant strain ME-XJ-8, an enhancement of 2,3-BD production up to 130 g/L could be obtained (Ji et al. 2010). Additionally, lower acetoin levels were obtained. The decrease in ethanol formation due to the inactivation of *aldA* led to an increase of the NADH:NAD^+ ratio. In the reversible reaction between acetoin and 2,3-BD, 2,3-BD production was favored by the additional NADH supply (Ji et al. 2010).

The insertional inactivation technology has also been successfully applied to reduce the lactic acid by-production during the co-production of 2,3-BD and 1,3-propanediol using *K. oxytoca* (Yang et al. 2007).

Furthermore, during cultivation using wild type *S. marcescens*, the surface-active exo-lipid serrawettin W1 was produced, which led to excessive foam formation. Therefore, a mutant of *S. marcescens* deficient in serrawettin W1 was constructed by inactivation of the *swrW* gene coding for serrawettin W1 synthase. This way, no addition of antifoam reagent would be necessary during cultivation (Zhang et al. 2010a; Ji et al. 2011).

Additional studies focused on strain adaptation to substrates that do not require pretreatment steps. An example is cloning the *malS* gene coding for α-amylase and over-expression of this enzyme in *K. pneumoniae*, thus enabling a one-step production of 2,3-BD from starch (Zheng et al. 2008).

Other attempts to increase 2,3-BD production were made by inserting the *vgb* gene encoding *Vitreoscilla* (bacterial) hemoglobin (VHb) into *E. aerogenes* and *S. marcescens*. This improves the physiological functionality during 2,3-BD production by allowing a better oxygen supply to the cells (Zhang et al. 2009).

Further studies focused on introducing gene fragments coding for key enzymes into heterologous hosts like *E. coli*, in order to produce enantio-pure 2,3-BD (Ji et al. 2011).

1.9 Objectives

The present thesis was performed within the framework of the EU-project called “Production and Upgrading of 2,3-Butanediol from Biomass” (PUBB). The aim of the PUBB project was the realization of an efficient bio-based process for the production of 2,3-BD from renewable ressources using non-pathogenic microorganisms.

The first task was the screening for non-pathogenic microorganisms capable of producing high amounts of 2,3-BD, comparable to risk class 2 strains. For the screening process, strains from culture collection and project partners as well as isolated microorganisms from environmental habitats were selected and their potential for 2,3-BD production was investigated.
In addition to cutivations using pure sugars, wood hydrolysates were employed as substrates for cultivation.
Furthermore, a strain improvement of the promising microorganisms was attempted by random mutagenesis.

After suitable non-pathogenic strains were selected, the process for 2,3-BD production was subjected to several optimization steps in the shake flask and bioreactor scale.

Initial optimizations were performed on medium containing pure sugars as carbon source in the shake flask scale.
Optimizations were carried out regarding medium composition and cultivation parameters. Optimum values for the initial sugar concentration, the nature of the nitrogen source, shaking rate and incubation temperature were determined during batch cultivations with free and immobilized cells.
Further investigations were performed using the fed-batch cultivation mode. The aim of the fed-batch cultivations was to start with the optimum initial glucose concentration and to reach maximum possible 2,3-BD production by stepwise feeding of nutrients.

Further investigations in the shake flask scale were conducted on wood hydrolysates. For the production of natural wood hydrolysates, many preliminary steps are required and the process is time consuming. Therefore, an artificial wood hydrolysate medium was constructed and employed for optimization purposes, after comparison to the corresponding natural hydrolysate medium.
Furthermore, the effect of potential inhibitory compounds present in natural wood hydrolysates on bacterial growth and 2,3-BD production was examined. The potential inhibitory compounds were tested separately or combined in various concentrations (1-fold, 2-fold, 4-fold, 8-fold and 16-fold).

A scale-up of 2,3-BD production to the 3.5 L bioreactor scale was carried out. The optimized parameters from the shake flask scale were employed and further optimization steps were carried out.
The experiments were conducted on medium with different C-sources: glucose, artificial wood hydrolysate and natural wood hydrolysate.
On artificial wood hydrolysate medium, cultivations were performed both with and without addition of potential inhibitory compounds.
The aim of the bioreactor experiments was to optimize the cultivation parameters (stirring speed, aeration rate, forced pH shifts) in order to reproduce the results obtained in the shake flask scale.
In order to increase 2,3-BD production, additional studies were carried out using the fed-batch cultivation mode.

After finding suitable settings for the cultivation parameters in the 3.5 L bioreactor scale, a further scale-up of 2,3-BD production to the 45 L bioreactor scale was attempted.
Several scale-up criteria were investigated regarding their suitability for a successful upscale of 2,3-BD production process.
The aim of this investigation was to reproduce the results obtained in the 3.5 L bioreactor scale.

2 Material and methods

2.1 Chemicals and reagents

All the chemicals used in the conducted experiments were purchased from one of the following companies unless otherwise indicated:

- Sigma-Aldrich Chemie GmbH (Steinheim, Germany)
- Carl Roth GmbH & Co. KG (Karlsruhe, Germany)
- AppliChem GmbH (Darmstadt, Germany)
- Merck KGaA (Darmstadt, Germany)
- Serva Electrophoresis GmbH (Heidelberg, Germany)

The antifoam reagent employed during bioreactor cultivation experiments was Ucolup N-115, marketed by Brenntag Mineralöl GmbH & Co. (Mülheim/Ruhr, Germany). The yeast extract was obtained from Ohly GmbH (Hamburg, Germany) and glucose from CG Chemikalien GmbH & Co. KG (Laatzen, Germany).

All solutions were prepared with Milli-Q water from EMD Millipore Corporation (Billerica, MA, USA).

DNS assay reagent

Tab. 2-1 shows the composition of the DNS assay reagent, which was used for the colorimetric detection of reducing sugars (see chapter 2.5.1.4). After use, the reagent was stored in a dark place.

Table 2-1 Composition of the DNS assay reagent

Chemical	Amount	
3,5-Dinitrosalicylic acid (DNS)	1	g
Potassium sodium tartrate	30	g
10% (w/v) NaOH in Milli-Q water	16	mL
Solve above in Milli-Q water	50	mL
Fill up with Milli-Q water to	100	mL

Modified Seebach solution as spray reagent for TLC

The modified Seebach solution (after Nicholson 2008) was used as spray reagent for the qualitative detection of 2,3-butanediol and acetoin by TLC (see chapter 2.5.1.5). The reagent was stored in a dark place after use. The composition of the reagent is listed in Tab. 2-2.

Tab. 2-2 Composition of the modified Seebach solution (Nicholson 2008)

Chemical	**Amount**
Phosphomolibdic acid	0.10 g
$(NH_4)_4Ce(SO_4)_4 \cdot 2H_2O$	0.05 g
Conc. H_2SO_4	0.60 mL
Fill up with Milli-Q water to	9.20 mL

2.2 Culture media

All culture media were sterilized by autoclaving for 20 min at 121 °C under 1 atm. The sugars, complex medium components and salt were sterilized separately and then mixed. The mineral solutions were prepared as stock solutions and the necessary amount was added to the medium after filter sterilization (0.45 µm Syringe Filter, Sartorius, Hamburg, Germany).

2.2.1 Medium for agar plate cultures

For the preparation of agar plates, commercial nutrient broth (NB) agar (Carl Roth GmbH & Co. KG, Karlsruhe, Germany) was used. After autoclaving, the medium was poured under sterile conditions into petri dishes (Ø 10 cm) and the plates were allowed to cool and solidify. The plates were stored at room temperature until use. The composition of the NB agar medium is listed in Tab. 2-3.

Tab. 2-3 Composition of the NB agar medium

Chemical	**Amount**
Nutrient agar for microbiology	23 g
- 5 g/L Peptone	
- 3 g/L Beef extract from gelatin	
- 15 g/L Agar	
Milli-Q water	fill up to 1000 mL

2.2.2 Medium for shake flask and bioreactor cultivations

For all shake flask and bioreactor experiments, the microorganisms were grown on the basal medium reported by Nakashimada et al. (1998). Tab. 2-4 shows the medium composition for 1 L containing 20 g/L glucose. For higher glucose concentrations multiple amounts were weighed in. The solutions 1, 2, 3 and 4 were mixed after being autoclaved separately, while the required amounts of solutions 5 and 6 were added after filter sterilization.

Tab. 2-4 Basal medium composition (with 20 g/L glucose) (after Nakashimada et al. 1998)

Solution	**Chemical**	**Amount [g]**	**Volume [mL]**
1	Glucose monohydrate	22.0	fill up to 300 mL
2	Yeast extract	5.0	fill up to 100 mL
	Tryptone	5.0	
3	K_2HPO_4	7.0	fill up to 400 mL
	KH_2PO_4	5.5	
4	$(NH_4)_2SO_4$	1.0	fill up to 180 mL
	$MgSO_4 \cdot 7H_2O$	0.25	
	$Na_2MoO_4 \cdot 2H_2O$	0.12	
	$CaCl_2 \cdot 2H_2O$	0.021	
5	$Co(NO_3)_2 \cdot 6H_2O$	0.29	fill up to 100 mL
	$(NH_4)_2Fe(SO_4)_2 \cdot 6H_2O$	0.39	add 10 mL to medium
6	Nicotinic acid	0.2	fill up to 1000 mL add 10 mL to medium
	$Na_2SeO_3 \cdot 5H_2O$	0.0262	
	$NiCl_2 \cdot 2H_2O$	0.0037	
	$MnCl_2 \cdot 4H_2O$	0.5	
	H_3BO_3	0.1	
	$AlK(SO_4)_2 \cdot 12H_2O$	0.0172	
	$CuCl_2 \cdot 2H_2O$	0.001	
	$Na_2EDTA \cdot 2H_2O$	0.554	

For the fed-batch experiments, all medium components (sugar, nitrogen source, salt) were added in solid form without previous sterilization. The trace elements were added in liquid form after sterile filtration. The feedings were carried out at different time points depending on the residual sugar concentration in the culture broth.

In addition to cultivations on pure sugars, further cultivations were performed using natural wood hydrolysates as sugar source. The hydrolysates were obtained from poplar wood chips after steam pretreatment and enzymatic hydrolysis and were delivered by our project partners from the Institute of Wood Research of the Thünen Institute. The initial pH value of the natural wood hydrolysates was in the range of 3.8-3.9; using solid NaOH the pH value was adjusted to 6.6. The other medium components were added in solid form (solutions 2-5 from Tab. 2-4), while solution 6 was added in liquid form. The medium was centrifuged for 30 min at 11,000 rpm and 4°C using a Heraeus Multifuge 1S-R equipped with a Sorvall SLA-3000 rotor (Thermo Scientific, West Palm Beach, FL, USA). The medium was filtered using a glass fiber prefilter (Whatman, Dassel, Germany) and a cellulose acetate filter (pore size 0.45 µm; Sartorius, Göttingen, Germany) and was then used for cultivation experiments.

2.3 Microbial strains

The microbial strains employed in this thesis were purchased from culture collections, mainly DSMZ (Braunschweig, Germany) and ATCC (Rockeville, Md., USA) or provided by project partners. These microorganisms and the respective sources are listed in Tab. 2-5.

Tab. 2-5 Microorganisms tested for 2,3-BD production and their provenience

Microorganism	Designation	Culture collection	Provenience
Paenibacillus polymyxa	ATCC 12321	ATCC	TU Braunschweig, Germany
	DSM 356	DSMZ	
	DSM 36	DSMZ	
Bacillus licheniformis	DSM 8785	DSMZ	
	ATCC 9789	ATCC	
Candida utilis	ATCC 22023	ATCC	
Klebsiella oxytoca*	ATCC 8724	ATCC	
	NRRL B-199	NRRL	
Klebsiella oxytoca*	m5a1	n.s.	Biopolis, Valencia, Spain
Raoultella planticola	CECT 843	CECT	
Enterococcus casseliflavus*	18.3	sludge isolate	
Lysinibacillus sphaericus	37	sludge isolate	
Lysinibacillus fusiformis	47	sludge isolate	
Bacillus licheniformis	TUL	n.s.	TU Lodz, Poland
Bacillus subtilis	TUL	n.s.	
Bacillus amyloliquefaciens	TUL	n.s.	

* risk group 2 strains

Paenibacillus polymyxa belongs to the Order *Bacillales*, Family *Paenibacillaceae*, *Bacillus licheniformis* to the Order *Bacillales*, Family *Bacillaceae*. Both bacterial strains are widely spread in soil. Further characteristics of the microorganisms are: Gram-positive, spore forming and facultative anaerobic. Additionally, these strains are non-pathogenic and were granted the GRAS (generally regarded as safe) status.

Some mutant strains were obtained by random mutagenesis using UV light from the wild type strains *Paenibacillus polymyxa* ATCC 12321 and *Bacillus licheniformis* DSM 8785. Furthermore, 12 microorganisms were isolated from natural habitats (Steinhuder Meer, Lower Saxony, Germany) and were also tested for their potential to produce 2,3-BD.

2.3.1 Culture preservation

For short-term storage, the microorganisms were streaked out on NB agar plates (composition listed in Tab. 2-4) and incubated for approx. 48 hours at 25°C (*Candida utilis*), 30°C (*P. polymyxa*, *Bacillus* sp.) or 37°C (*K. oxytoca*, *R. planticola*, *E. casseliflavus*, *Lysinibacillus* sp.). Afterwards the plates were stored at 4°C. The microorganisms were inoculated onto new plates every 2-4 weeks.

For long-term conservation, glycerol stock cultures were prepared. 230 µL of a 24-h shake flask culture were mixed with 770 µL 80% (v/v) glycerol in 2 mL cryo vials, which were then stored in liquid nitrogen.

2.3.2 UV mutagenesis

2,3-BD producing microbial strains were subjected to random mutagenesis using UV light. The procedure followed several steps. In the first step, the strain was cultivated on 100 mL medium in 500 mL shake flasks at 30°C and 100-150 rpm until an OD value of 8-10 was reached. Afterwards, growth was stopped by putting the culture on ice for 10 min. 1 mL sample was taken and centrifuged for 3 min at 10,000 rpm; the supernatant was discarded and the pellet was resuspended in 1 mL 0.1 M $MgSO_4$ solution. The suspension was diluted in a ratio of 1:1000 with 0.1 M $MgSO_4$ solution. Diluted suspension was filled into petri dishes (6 cm diameter; 3 mL for each dish) and subjected to UV-mutagenesis at 260 nm for 30-120 seconds. 100 µL suspension from each petri dish was plated onto agar plates and incubated at 30°C for 24-72 h. Potential mutant colonies were picked from the agar plates and cultured in 96-well microtiter plates on 200 µL medium containing 0.04 g/L bromcresolgreen at 25-30°C for 24 h. Mutants from wells showing weak or no colour change were isolated onto new agar plates and their potential for 2,3-BD production was tested in the shake flask scale.

2.3.3 Isolation of microorganisms from environmental samples

For the isolation of microorganisms from environmental samples selective agar plates were prepared. The composition of the medium is given in Tab. 2-6. Solution II was autoclaved and after cooling to 60°C, the sterile filtered solution I containing the selective compounds of the medium was added. The medium was poured under sterile conditions into petri dishes (Ø 10 cm) and the plates were allowed to cool and solidify.

Various environmental samples (water, driftwood, addled wood) were collected from the Steinhuder Sea (Lower Saxony, Germany). The wood samples were chopped under sterile conditions, transferred to 1.5 mL Eppendorf microtubes and 1 mL 0.1 M $MgSO_4$ solution was added. After 2 h at 25°C, the supernatant was removed using a micropipette and filled into a fresh Eppendorf microtube. 100 µL supernatant (or 100 µL water sample) was plated onto

selective agar plates and incubated for 4 d at 25°C. Colonies were picked and the microorganisms were isolated on new culture plates, which were renewed every 2-4 weeks.

Tab. 2-6 Composition of the medium for selective agar plates

Solution	**Chemical**		**Amount**	
I	Natural wood hydrolysate (containing 12.85 g glucose)		400.0	mL
(sterile filtered)	Urea		1.365	g
	Amphotericin B		0.025	g
II	Agar		7.5	g
(autoclaved)	Milli-Q water	fill up to	100.0	mL

2.4 Culture conditions

2.4.1 Precultures

All inocula were prepared using 500 mL Erlenmeyer flasks with 2 baffles. 100 mL culture medium (containing 20 g/L glucose) was incubated with a loop full of cells from agar plate cultures at 30°C and 100 rpm for approx. 24 hours until an OD value of 8-10.

For cultivations performed in the bioreactor scale, several precultures were prepared under the same conditions. In the 3.5 L bioreactor scale (3 L working volume), 200 mL preculture were employed for inoculating the culture medium; 2 L preculture were required for 45 L bioreactor scale (30 L working volume).

For bioreactor cultivations on natural wood hydrolysates, the inoculum was prepared in two steps. In the first step, a preculture on glucose medium was performed. 5 mL of this preculture were transferred to 95 mL natural wood hydrolysate in 500 mL Erlenmeyer flasks and incubated until an OD value of 20 was reached. The second preculture was employed to inoculate the wood hydrolysate medium in the 3.5 L bioreactor.

2.4.2 Shake flask cultivations

Cultivation experiments were carried out in 500 mL Erlenmeyer flasks with 2 baffles and 100 mL working volume. The basal medium was inoculated from 24 h-old precultures. The amount of inoculum was calculated so as to reach an initial optical density (540 nm) of 0.4. Initial medium pH ranged from 6.5 to 6.8 and remained unadjusted during cultivation. The cultures were incubated under different conditions in order to identify the optimum cultivation parameters until the entire sugar was consumed.

For analyzing the influence of potential inhibitory compounds present in wood hydrolysates, cultivations were carried out in 100 mL Erlenmeyer flasks with 2 baffles and 20 mL working volume. During these experiments, the potential inhibitory compounds were added to the culture medium in various concentrations and growth as well as sugar consumption and 2,3-BD production were assayed.

2.4.3 Cultivations in the 3.5 L bioreactor scale

Bioreactor cultivations in the 3.5 L scale were performed using the Minifors reactors (Infors AG, Bottmingen, Switzerland). The general layout of the bioreactor is shown in Fig. 2-1.

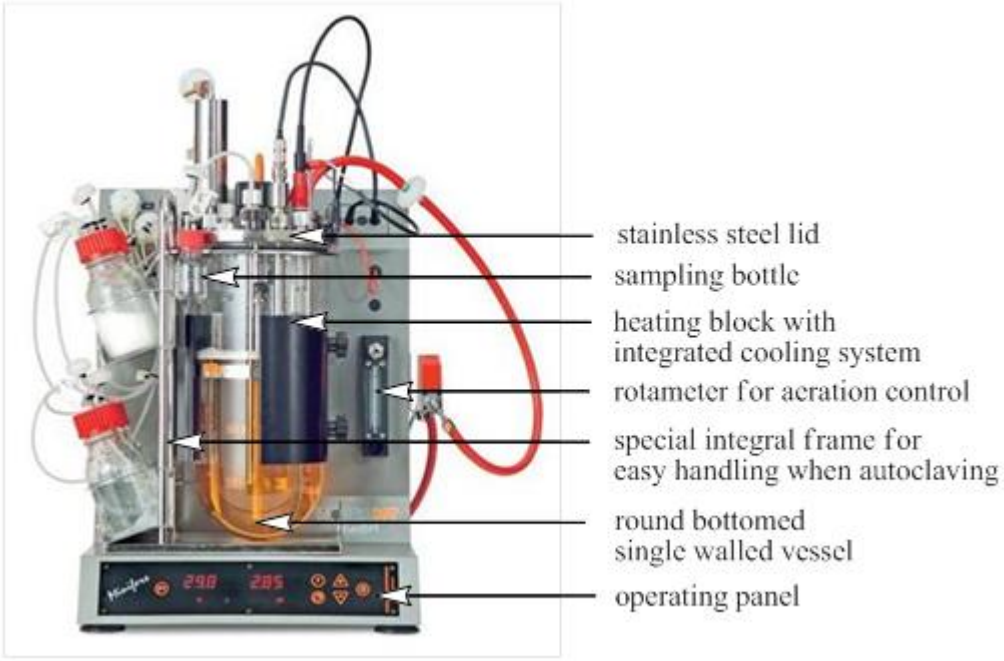


Figure 2-1 Front view of the 3.5 L Minifors bioreactor (modified after www.infors-ht.com)

The round-bottomed single walled vessel consists of borosilicate glass covered by a stainless steel lid. The bioreactors lid has ports for electrodes (pH, pO_2, OD, antifoam, temperature), ports for the supply of feeding nutrients, acids/bases for pH adjustment or antifoam reagent, a port for the connection to an exit gas cooler and a sampling device (Fig. 2-2). The vessel, bottles and pump heads are all supported in a special integral frame for easy handling when autoclaving. Furthermore, 2 sensors for measurement of O_2 and CO_2 amounts in the exhaust gas were connected to the bioreactor. The stirrer system consisted of two Rushton turbines (6 blade disk impellers).

The regulation of the standard measuring and control parameters was carried out by a direct digital control (DDC) system, whose operating panel is integrated into the base of the fermenter. The operating panel enabled easy setting of key parameter values along with calibration and various control options. The following main parameters were observed and/or controlled during fermentation: stirring speed, temperature, pH, pO_2, antifoam level, aeration

control (gas supply) and feed. The online data acquisition was provided by the IRIS fermenter control system software.

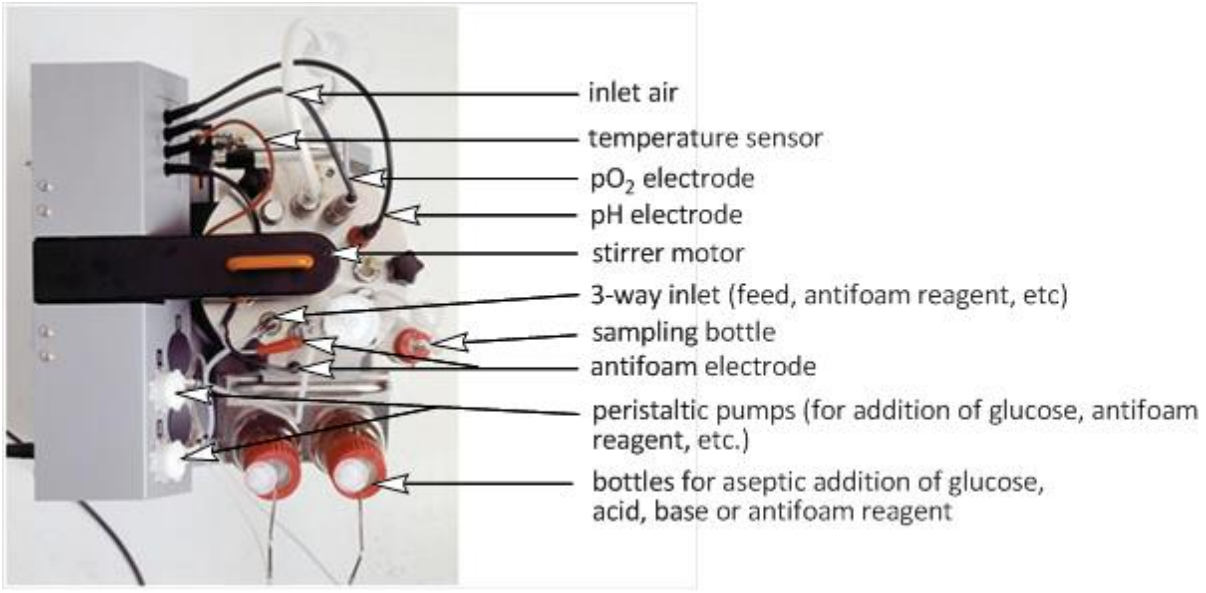


Figure 2-2 Top view of the 3.5 L Minifors bioreactor (modified after www.infors-ht.com)

The working volume for all cultivations was 3 L; the amount of culture medium 2.8 L. Solution 1 containing the sugar source was filled into the bioreactor and autoclaved, while the other medium components were pumped into the bioreactor after cooling down. The culture medium was inoculated with 200 mL preculture. For the temperature, optimized values from the shake flask experiments were used. The first cultivations were conducted using a stirring speed of 200 rpm and an aeration rate of 0.5 L/(L*min), based on data from literature (Nakashimada et al., 2000). Both parameters were optimized during further experiments.

2.4.4 Cultivations in the 45 L bioreactor scale

For the scale-up of cultivations experiments to the 45 L scale, a stainless steel stirred tank bioreactor from the company Sartorius Stedim Systems GmbH (formerly B. Braun Melsungen AG, Melsungen, Germany) was employed. The bioreactor was equipped with a thermostat, a sensor for antifoam monitoring and automatic addition of antifoam reagent, three Rushton turbines, a flow meter for adjusting the inlet air flow, an exit gas cooler, a pressure relief valve, two sensors for exhaust air analysis and sensors for pH and pO_2 measurement.

After calibration of the pH electrode, the sugar source was added in solid form to the bioreactor, which was then filled with the desired amount of distilled water. The bioreactor containing the sugar solution (solution 1) was sterilized using the standardized sterilization program of the reactor. After cooling down, the solutions containing all other medium components were added to the reactor, leading to a total volume of culture medium of 28 L. The calibration of sensors for pO_2-measurement and exhaust air analysis was performed and the culture medium was

inoculated with 2 L preculture. The values for stirring speed, temperature and aeration rate were set using the control panel of the bioreactor.

2.5 Analytical procedures

During shake flask and bioreactor cultivation experiments samples were taken every 2-6 h and the progress of the cultivation was monitored by performing a series of offline analytical procedures. Additional monitoring of bioreactor cultivations was achieved by the use of online sensors.

2.5.1 Offline analytics

Offline analytical procedures applied during shake flask and bioreactor cultivations in this thesis include:

- the determination of cell growth by measurement of optical density (OD) and/or cell dry weight (CDW)
- pH measurements
- DNS-assay for reducing sugars
- Thin layer chromatography (TLC) for qualitative detection of 2,3-BD and acetoin production
- High performance liquid chromatography (HPLC) for quantitative measurement of sugar consumption as well as 2,3-BD and by-product formation during cultivation.

2.5.1.1 Optical density (OD)

Cell growth during cultivation experiments was primarily monitored by measuring the turbidity (optical density) of the culture broth directly after sampling. The OD measurements were performed at 540 nm using an Ultraspec 3000 spectrophotometer (Pharmacia Biotech, Amersham, Sweden). Samples were diluted with Milli-Q water so that the absorbance was between 0.1 and 0.7 units. The corresponding culture medium (same dilution ratio as for the samples) was used as reference.

2.5.1.2 Cell dry weight (CDW)

Due to limited sample volume, cell dry weight was estimated for most cultivations using a correlation between optical density and cell dry weight. In order to establish this correlation, several shake flask cultivations were performed in parallel under the same conditions. Each flask was used for the determination of both OD (at 540 nm) and CDW at a different cultivation time point.

For CDW measurement, 20 mL culture broth were added to cooled, pre-weighed metal centrifuge tubes, which were then centrifuged for 30 min at 13,000 rpm. The supernatant was discarded; the pellet was washed twice with 20 mL Milli-Q water and then dried for 48 h at 80°C. Afterwards, the centrifuge tubes were left to cool, weighed again and CDW was calculated.

According to the correlations, one OD unit (540 nm) was estimated as being equivalent to 0.28 g/L cell dry weight for *B. licheniformis* DSM 8785 and 0.32 g/L cell dry weight for *P. polymyxa* ATCC 12321.

2.5.1.3 pH measurement

Offline pH measurement of culture samples was performed using a digital pH-meter (CG 802, Schott, Mainz, Germany). In order to ensure the accuracy of the pH electrode, a two-point calibration with buffer solutions of pH 4 and pH 7 was conducted on a daily basis.

2.5.1.4 DNS assay

Glucose consumption during cultivation can be monitored using the DNS assay, a colorimetric test for the determination of reducing sugars and other reducing compounds. In the presence of reducing sugars or other reducing compounds, 3,5-dinitrosalicylic acid is reduced to 3-amino-5-nitrosalicylic acid (Fig. 2-3). During this reaction, the color of the sample shifts from yellow-orange to red and an increase in the absorbance at 540 nm occurs. Using a calibration line, the amount of glucose in the sample can be determined.

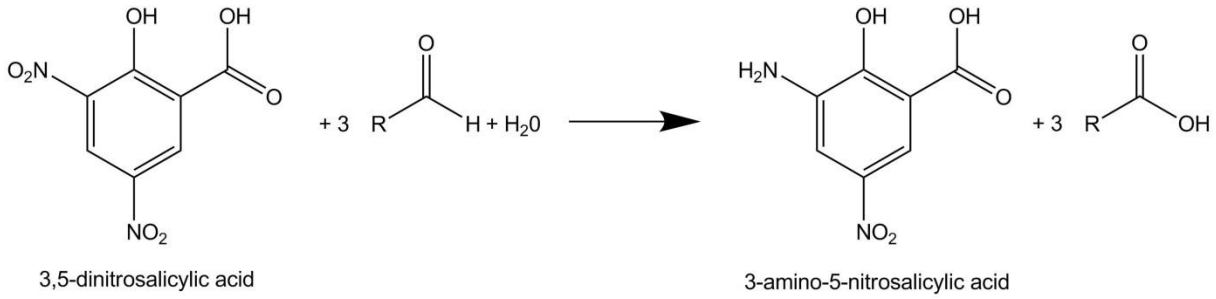


Figure 2-3 Reaction of 3,5-dinitrosalicylic acid with reducing compounds

For the measurement, 100 µL DNS reagent (composition is given in Tab. 2-1, chapter 2.1) were mixed with 100 µL culture supernatant and incubated for 7 min at 95°C using a thermo shaker (Thermostat 5320, Eppendorf, Germany). The mix was left to cool; 800 µL Milli-Q water were added, the solution was mixed and the absorbance was measured at 540 nm using a spectrophotometer.

In addition to reducing sugars (e.g. glucose), other reducing compounds like acetoin can also react with 3,5-dinitrosalicylic acid (Hespell 1996)

.

2.5.1.5 Thin layer chromatography (TLC)

Thin layer chromatography is a simple, quick and inexpensive chromatographic technique used for separating and identifying organic compounds from a mixture. This technique requires a stationary phase, a thin layer of adsorbent material (usually silica gel or aluminium oxide), immobilized on a support (aluminium foil, glass or plastic sheet), and a solvent (mobile phase). TLC consists of three steps: spotting, development and visualization. The samples are applied to a TLC plate as small spots, about 1.5 cm from the bottom of the plate. The TLC plate is then placed in a solvent reservoir and the mobile phase is drawn up the plate by capillary action. The different components in the mixture are carried up the plate at different rates due to their different polarities and their solubility in the solvent. When the solvent front almost reaches the top of the thin layer, the plate is removed from the solvent reservoir. After drying, the chromatogram is colored with a spray reagent for visualization. The TLC plate is heated at 95°C and the spots corresponding to the compounds appeared on the silica gel layer. The constituents of a sample can be identified by running unknown and internal standard samples simultaneously.

In this thesis, TLC was used for a qualitative detection of 2,3-BD and acetoin production during cultivation. The employed TLC plates were Alugram® Sil G (Macherey-Nagel, Düren, Germany) with a thin layer of silica gel 0.2 mm as stationary phase. 5 µL supernatant from culture samples were spotted on the TLC plate. The mobile phase consisted of n-heptane/ethylacetate/ethanol in a ratio of 1:5:1 (v/v/v). As spray reagent for the visualization of 2,3-BD and acetoin, the modified Seebach solution described by Nicholson (2008) was employed (the composition is given in Tab. 2-1, chapter 2.1). Fig. 2-4 shows a thin layer chromatogram of acetoin and 2,3-BD standard solutions (2 g/L – 20 g/L).

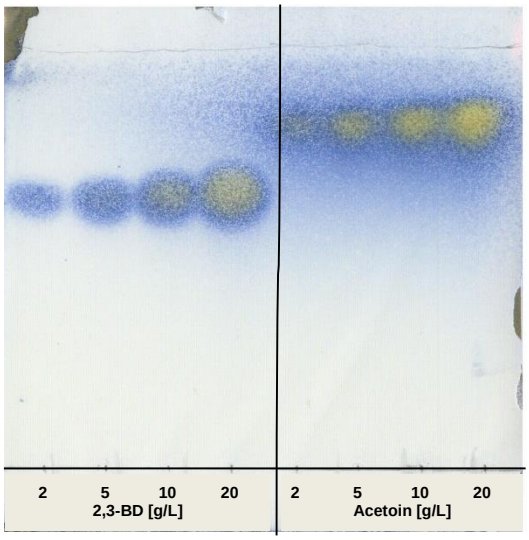


Figure 2-4 TLC of 2,3-BD and acetoin standard solutions; 5 µL sample volume; solvent sytem: n-heptane:ethylacetate:ethanol (1:5:1) (v/v/v)

2.5.1.6 High performance liquid chromatography (HPLC)

High performance liquid chromatography is a method used to separate, identify and quantify various compounds present in a mixture. The sample is pumped together with the mobile phase and carried through the analytical column, which is packed with the stationary phase. The components of a mixture can be separated based on their different interaction strength with the stationary phase of the column. The compounds leave the analytical column at different time points (retention time) and are characterized by using RI- or UV-detectors.

HPLC was used in this thesis for the detection and quantification of the sugar source (glucose, xylose) used for cultivation as well as the fermentation main product (2,3-BD) and by-products (e.g. acetoin, glycerol, acetate, ethanol). For detection the analytical column Aminex® HPX-87H column (300x7.8 mm) from the BioRad Laboratories (California, USA) was employed. The stationary phase contained by the column is a styrene divinylbenzene resin (8% crosslinked; ionic form). Compound separation is based on a combination of reverse phase and ion exclusion principles. A SecurityGuard Cartridge Carbo-H 4x3.0 mm (Phenomenex, Aschaffenburg, Germany) was installed in front of the analytical column in order to remove impurities. The column was operated at 60°C (for samples containing glucose) and 25°C (for samples containing sucrose) using a LaChrom L-7300 column oven (Merck-Hitachi, Germany). As mobile phase, 0.005 M sulfuric acid diluted from 1 M stock solution (Carl Roth, Karlsruhe, Germany) was used. The flow rate for the mobile was adjusted to 0.5 mL/min (0.4 mL/min for sucrose samples). A refractive index (RI) detector Ri 71 from Shodex (Germany) and an UV detector (210 nm; Techlab, Germany) were employed for detection. The pumping system 880Pu was purchased from Jasco (Gross-Umstadt, Germany), the Basic Marathon autosampler from Spark Holland (Emmen, The Netherlands).

Before HPLC measurement, samples were centrifuged for 10 minutes at 13,000 rpm. The supernatant was filtered through a syringe filter (pore size 0.22 µm) and filled into HPLC sample vials. Samples containing amounts of glucose above 50 g/L were diluted with mobile phase. Data analysis was performed using the software Clarity (DataApex Ltd, Prague, Czech Republic).

The compounds were identified after calibration with self-prepared standard solutions. The Aminex HPX-87H column allows a good differentiation of *meso*-2,3-BD from the optically active stereoisomers (*D-(-)* and *L-(+)*) of 2,3-BD. However, a distinction between *D-(-)*- and *L-(+)*-2,3-BD production by this method is not possible. Fig. 2-5 shows a chromatogram of standard solutions of glucose, xylose, 2,3-BD and selected fermentation by-products.

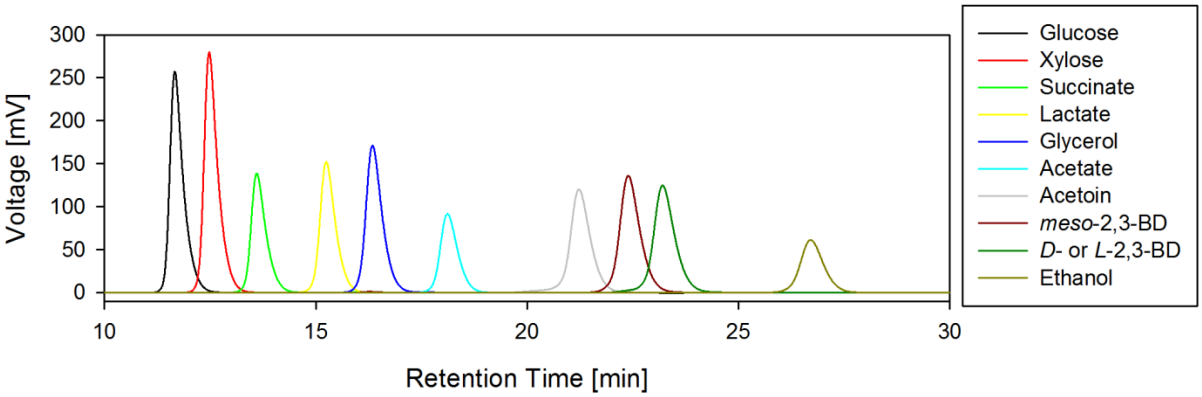


Figure 2-5 HPLC diagram of sugars, 2,3-BD and by-products (standard solutions; 10 g/L each)

2.5.2 Online analytics

During bioreactor experiments, additional monitoring of the cultivation progress was achieved by the use of sensors for the online data acquisition of pH, oxygen partial pressure (pO_2), biomass, O_2 and CO_2 amounts (exhaust gas analysis).

2.5.2.1 pH measurement

For the pH monitoring during bioreactor cultivation, a Sen Tix thermally stable pH electrode (WTW, Weilheim, Germany) was applied. It was calibrated before autoclaving using standard solutions of pH 4 and 7.

2.5.2.2 Oxygen partial pressure (pO_2)

The oxygen partial pressure was measured using a VISIFERM DO sterilizable optical sensor from the company Hamilton Messtechnik GmbH (Höchst im Odenwald, Germany). The measurement principle is based on photobleaching. Electrons are excited to a higher energy level by the blue light generated by sensor. They return back to their ground state after emission of red light. In the presence of oxygen, the luminophore comes into contact with oxygen, the oxygen molecule absorbs the energy and leads to a reduced intensity of red light emission (photobleaching). The intensity difference, thus oxygen concentration, is analyzed by the built-in sensor system. By employing optical oxygen sensors, a higher resolution, a shorter response time and a higher precision are achieved, compared to using amperometric sensors. The electrode was sterilized in the bioreactor and the oxygen partial pressure measured before the inoculation was defined as 100%. pO_2 can be expressed in %-form or concentration (mg/L, ppm, ppb). In this thesis, the pO_2 value was displayed as % air saturation. The calibration was performed after all culture parameters (temperature, aeration rate, pH, stirrer speed and culture volume) were stable.

2.5.2.3 Online biomass monitoring

The online biomass monitoring during cultivation was achieved using the evo 200 system from the company FOGALE nanotech (Nimes, France). The biomass detection sytem consists of an OD probe (Biomass Capacitance sensor DN12/220), a preamplifier for the OD sensor and the evo 200 control unit for data acquisition.

2.5.2.4 Exhaust gas analysis

During bioreactor cultivations, the amount of oxygen and carbon dioxide in the exhaust air was determined by the use of two sensors (BlueSens gas sensors GmbH, Herten, Germany), installed directly in the exhaust gas line. The measuring principle of the O_2 sensor is the ZrO_2 technology; the CO_2 sensor operates according to the infrared Technology with dual wavelength.

The O_2- and CO_2-sensor were calibrated before inoculation after all culture parameters (temperature, aeration rate, pH, stirrer speed and culture volume) were stable. During cultivation, the sensors transmitted the data to a central computer, where it was collected by the software FERMVIS and the values for oxygen uptake rate (OUR), carbon dioxide evolution rate (CER) and respiratory quotient (RQ) were calculated.

Calculation of these parameters is based on the method of the quasi steady-state balance of inert gas and is shown in following equations (Rau & Graßl 2009):

$$OUR = \frac{V'^N \cdot p^N \cdot X^E_{O2} \cdot M_{O2}}{V_F \cdot R \cdot T^N} \cdot \left[1 - \frac{X^A_{O2}\left(1 - X^E_{O2} - X^E_{CO2}\right)}{X^E_{O2}\left(1 - X^A_{O2} - X^A_{CO2}\right)}\right] \quad \textbf{(Equation 2-1)}$$

$$CER = \frac{V'^N \cdot p^N \cdot X^E_{CO2} \cdot M_{CO2}}{V_F \cdot R \cdot T^N} \cdot \left[\frac{X^A_{CO2}\left(1 - X^E_{O2} - X^E_{CO2}\right)}{X^E_{CO2}\left(1 - X^A_{O2} - X^A_{CO2}\right)} - 1\right] \quad \textbf{(Equation 2-2)}$$

$$RQ = \frac{CER \cdot M_{O2}}{OUR \cdot M_{CO2}} \quad \textbf{(Equation 2-3)}$$

where:
- T^N = standard temperature = 273.15 [K];
- p^N = standard pressure = 1 [atm] = 101 325 [Pa] = 1.0133 [bar];
- R = universal gas constant = 8.314 [J/ mol · K] = $8.314 \cdot 10^{-2}$ [barL/ molK];
- X^E_{O2} = mole fraction of O_2 in the inlet air = 0.20946;
- X^E_{CO2} = mole fraction of CO_2 in the inlet air = 0.00033;
- X^A_{O2} = mole fraction of O_2 in the exhaust air (outlet);
- X^A_{CO2} = mole fraction of CO_2 in the exhaust air (outlet);
- V_F = volume of the fluid in the bioreactor;
- V'^N = volume stream under standard conditions [h^{-1}];
- M_{O2} = molar mass of O_2 = 31.998 g/mol;
- M_{CO2} = molar mass of CO_2 = 44.008 g/mol.

3 Results

The present thesis was performed within the framework of the EU-project called “Production and Upgrading of 2,3-Butanediol from Biomass” (PUBB). The main tasks for our Institute within the PUBB project were:

- Screening for 2,3-BD producing microorganisms and strain improvement by random mutagenesis;
- Optimization of medium components and cultivation parameters for 2,3-BD production with selected strains;
- Cultivations on natural wood hydrolysate and study of the effect of potentially inhibitory compounds present in the hydrolysates;
- Scale-up of 2,3-BD production process.

Some parts of the results presented in this thesis are joint work with the biotechnology students working on the PUBB project, due to the large time investment required for the cultivation experiments. For the data acquisition, a strict supervision of the cultures on a 24 hour basis, 5-7 days per week, was necessary, combined with frequent sampling, sample analysis and interpretation as well as varying feeding time points and extensive feeding procedures for fed-batch experiments depending on the residual sugar concentration.

3.1 Screening for 2,3-BD producing microorganisms

The first task of this thesis was the screening for non-pathogenic microorganisms capable of producing high amounts of 2,3-BD, comparable to risk class 2 strains. For the screening process, strains from culture collection and project partners as well as isolated microorganisms from environmental habitats were selected and their potential for 2,3-BD production was investigated.

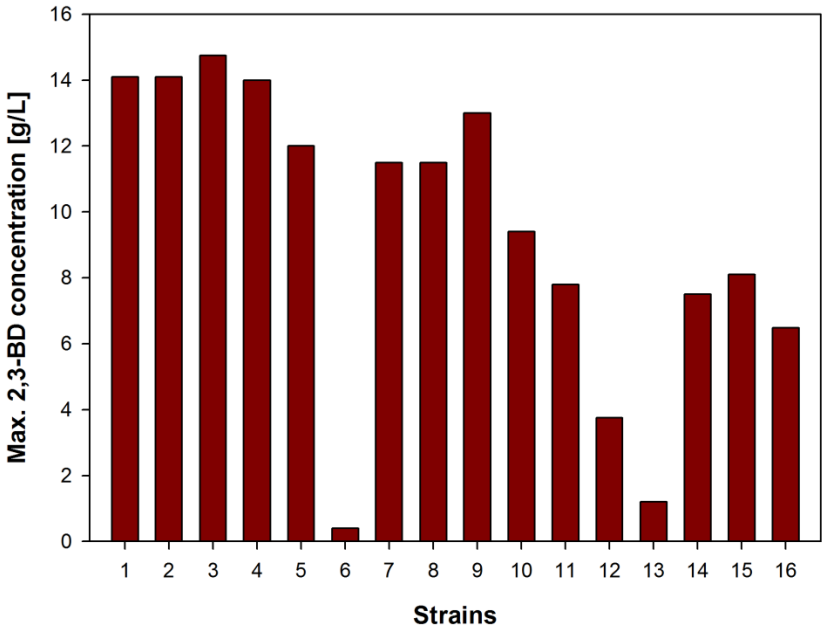
3.1.1 Wild type strains from culture collections and project partners

The screening was started with commercially available strains possessing the potential to produce 2,3-BD according to data from literature. The main focus was on non-pathogenic strains (belonging to the risk group 1); additionally, risk group 2 microorganisms were employed as reference for 2,3-BD production.

Eight microbial strains were purchased from the culture collections DSMZ (German Collection of Microorganisms and Cell Cultures), ATCC (American Type Culture Collection) and NRRL (Northern Regional Research Laboratory). Six of the purchased microorganisms were non-pathogenic (*Paenibacillus polymyxa*, *Bacillus licheniformis*, *Candida utilis*), while two strains

belonged to the risk group 2 (*Klebsiella oxytoca*). Further eight strains were received from partner institutions working on the PUBB project: Biopolis S.L. (Valencia, Spain) and Technical University Lodz (Lodz, Poland).

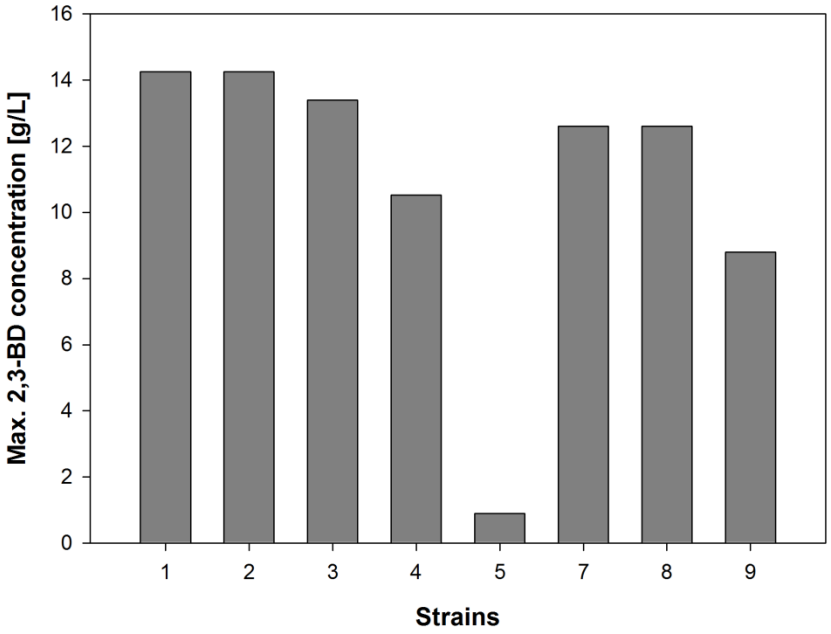
All sixteen microorganisms and their respective sources are listed in Tab. 2-5 (Chapter 2.3). The strains were inoculated on medium containing 30 g/L glucose and incubated in shake flasks at 30°C and 100 rpm. Fig. 3-1 shows a comparison of the maximum 2,3-BD concentrations reached during cultivation with these strains. The best 2,3-BD production from 30 g/L glucose was reached with the non-pathogenic strains *P. polymyxa* (strains no.1-3 from Fig. 3-1) and *B. licheniformis* (strains no. 4-5). The product concentrations for these strains ranged from 12 to 14.75 g/L, while risk group 2 *K. oxytoca* (strains no. 7-9) showed 2,3-BD amounts between 11.5-13 g/L. All other microorganisms (strains no. 6 and 10-16) yielded amounts of 2,3-BD lower than 10 g/L.



No.	Strain
1	*P. polymyxa* ATCC12321
2	*P. polymyxa* DSM 356
3	*P. polymyxa* DSM 36
4	*B. licheniformis* DSM 8785
5	*B. licheniformis* ATCC 9879
6	*C. utilis* ATCC 22023
7	*K. oxytoca* ATCC 8724
8	*K. oxytoca* NRRL B-199
9	*K. oxytoca* m5a1
10	*R. planticola* CECT 843
11	*E. casseliflavus* 18.3
12	*L. sphaericus* 37
13	*L. fusiformis* 47
14	*B. licheniformis* TUL
15	*B. subtilis* TUL
16	*B. amyloliquefaciens* TUL

Figure 3-1 Maximum 2,3-BD production of the screened strains on medium with 30 g/L glucose. Conditions: 500 mL shake flasks, 100 mL medium, 30 °C, 100 rpm, initial pH 6.6 (not adjusted), 15-38.5 h

The best producers (strains no. 1-5 and 7-9 from Fig. 3-1) were selected for further testing on wood hydrolysate medium. The selected strains were cultivated on the natural wood hydrolysate medium containing 30 g/L glucose (30°C, 100 rpm) and the maximum 2,3-BD amounts reached are shown in Fig. 3-2. The highest 2,3-BD production on natural wood hydrolysate medium containing 30 g/L glucose was reached with *P. polymyxa* (strains no.1-3) and *B. licheniformis* DSM 8785. These microorganisms yielded amounts of 2,3-BD between 10.5-14.3 g/L, while *B. licheniformis* ATCC 9879 showed product concentrations below 1 g/L. In comparison to the non-pathogenic strains, risk group 2 *K. oxytoca* (strains no. 7-9) produced 8.8-12.6 g/L 2,3-BD.



No.	Strain
1	*P. polymyxa* ATCC12321
2	*P. polymyxa* DSM 356
3	*P. polymyxa* DSM 36
4	*B. licheniformis* DSM 8785
5	*B. licheniformis* ATCC 9879
7	*K. oxytoca* ATCC 8724
8	*K. oxytoca* NRRL B-199
9	*K. oxytoca* m5a1

Figure 3-2 Maximum 2,3-BD production of selected strains on natural wood hydrolysate medium (containing 30 g/L glucose). Conditions: 500 mL shake flasks, 100 mL medium, 30 °C, 100 rpm, initial pH 6.6 (not adjusted), 20-60.5 h

P. polymyxa ATCC 12321, DSM 356 and DSM 36 and *B. licheniformis* DSM 8785 were subjected to additional cultivation tests on glucose medium with increased sugar content. The glucose concentration in the medium was raised to 60 g/L and cultures were incubated at 30°C and 100 rpm. The strains were compared concerning 2,3-BD production, yield and productivity and the results are summarized in Tab. 3-1. Maximum 2,3-BD concentrations and yields were in the same range for three of the strains (*P. polymyxa* ATCC 12321, DSM 356 and *B. licheniformis* DSM 8785). Furthermore, *P. polymyxa* ATCC 12321 and *B. licheniformis* DSM 8785 reached productivities of 0.74 and 0.87 g/(L*h), respectively. For *P. polymyxa* DSM 356 the productivity was much lower, reaching a value of 0.34 g/(L*h).

Table 3-1 Comparison of maximum 2,3-BD production, yield and productivity during cultivation of selected strains on medium with 60 g/L glucose. Conditions: 500 mL shake flasks, 100 mL medium, 30 °C, 100 rpm, initial pH 6.6 not adjusted, 25-69 h

Strain	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]
P. polymyxa ATCC 12321	**22.6**	**0.38**	**0.74**
P. polymyxa DSM 356	**23.5**	**0.39**	0.34
P. polymyxa DSM 36	17.2	0.29	0.65
B. licheniformis DSM 8785	**21.8**	**0.36**	**0.87**

Based on these results, *P. polymyxa* ATCC 12321 and *B. licheniformis* DSM 8785 were used for further studies and optimization procedures.

3.1.2 Strain improvement by random mutagenesis

In order to increase 2,3-BD production, a strain improvement of the selected strains (*P. polymyxa* ATCC 12321 and *B. licheniformis* DSM 8785) was attempted by random mutagenesis using UV light (260 nm). 2,3-BD is produced during the mixed acid fermentation pathway, with acids (e.g. lactic acid, acetic acid) as main by-products. The principle for mutant selection was isolating those mutants showing low acid production, because they could lead to increased concentrations of 2,3-BD.

Potential mutants from *P. polymyxa* ATCC 12321 were cultivated in 96-well microtiter plates. The selection was performed by adding the pH-indicator bromocresol green to the cultivation medium. The pH of the medium is 6.6, showing a dark blue colour in the presence of the pH indicator. If during cultivation acids are produced, the pH of the culture decreases and the color shifts to light blue and finally yellow. An example of 96-well microplate cultures of potential UV-mutants of *P. polymyxa* ATCC 12321 is shown in Fig. 3-3. The positive control corresponds to wild type strain cultures and the negative control to pure culture medium.

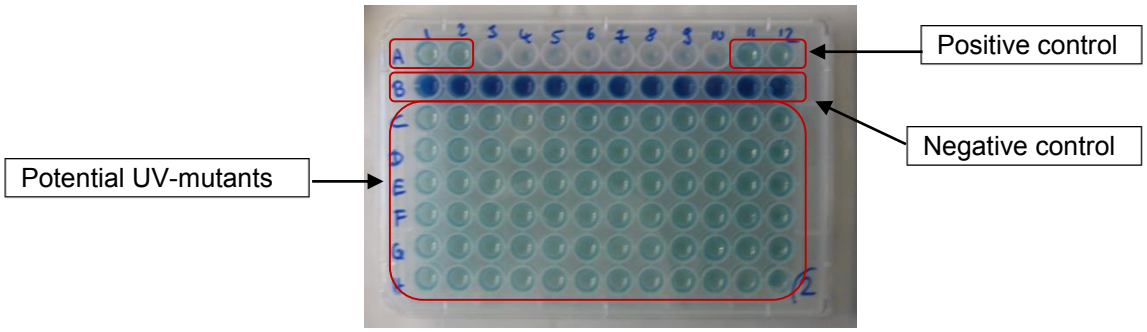


Figure 3-3 Example of 96-well microplate cultures: positive and negative controls, potential UV-mutants. Conditions: 200 µL medium containing bromecresolgreen, 25°C, 48 h [joint work with Iphöfer, 2011]

44 potential UV-mutants showing low or no color shift were detected. 2,3-BD production by the potential mutants was tested using TLC analysis (see Appendix, Fig. 9-1); 38 potential mutants showing 2,3-BD production were isolated on agar plates and then cultivated in shake flasks on medium with 50 g/L glucose at 35°C and 150 rpm. Results are summarized in Tab. 3-2. The cultivation of potential UV-mutants on glucose medium showed that all selected mutants produced lower amounts of 2,3-BD compared to the wild type strain.

Further experiments presented in this thesis with *P. polymyxa* ATCC 12321 were performed using the wild type strain.

Table 3-2 Summarized results of 2,3-BD production by potential UV-mutants of *P. polymyxa* ATCC 12321. Conditions: 500 mL shake flasks, 100 mL medium (50 g/L glucose), 35°C, 150 rpm, 10 h. wt = wild type strain *P. polymyxa* ATCC 12321 [joint work with Iphöfer, 2011]

No.	2,3-BD [g/L]	No.	2,3-BD [g/L]	No.	2,3-BD [g/L]	No.	2,3-BD [g/L]
1	4.41	11	4.30	24	2.87	34	9.05
2	5.27	12	5.42	25	3.82	35	8.93
3	2.19	16	2.98	26	3.13	**36**	**10.89**
4	2.40	17	0.96	27	3.71	**37**	**9.59**
5	3.26	18	2.04	28	5.41	**38**	**9.13**
6	4.18	19	1.33	29	5.08	39	8.57
7	5.21	20	1.94	30	4.47	**41**	**9.81**
8	3.96	21	0.49	31	5.33	**42**	**10.99**
9	2.63	22	1.42	32	3.02		
10	5.02	23	0.90	33	7.72	wt	15.47

For strain improvement of *B. licheniformis* DSM 8785, potential UV-mutant selection using a pH indicator was not possible. During cultivation with the wild type strain, the pH did not decrease enough as to induce a color shift of the culture broth. Therefore only the selection step by TLC-analysis was performed for the potential mutants. 59 potential mutants of B. liceniformis DSM 8785 from 96-well-plate cultivation on glucose (20 g/L) were tested on 2,3-BD production by TLC (see Appendix, Fig. 9-2). 20 of these potential mutants showing stronger spots on the TLC were selected on agar plates and then cultivated in the shake flask scale on medium with 120 g/L glucose at 30°C and 100 rpm (Fig. 3-4).

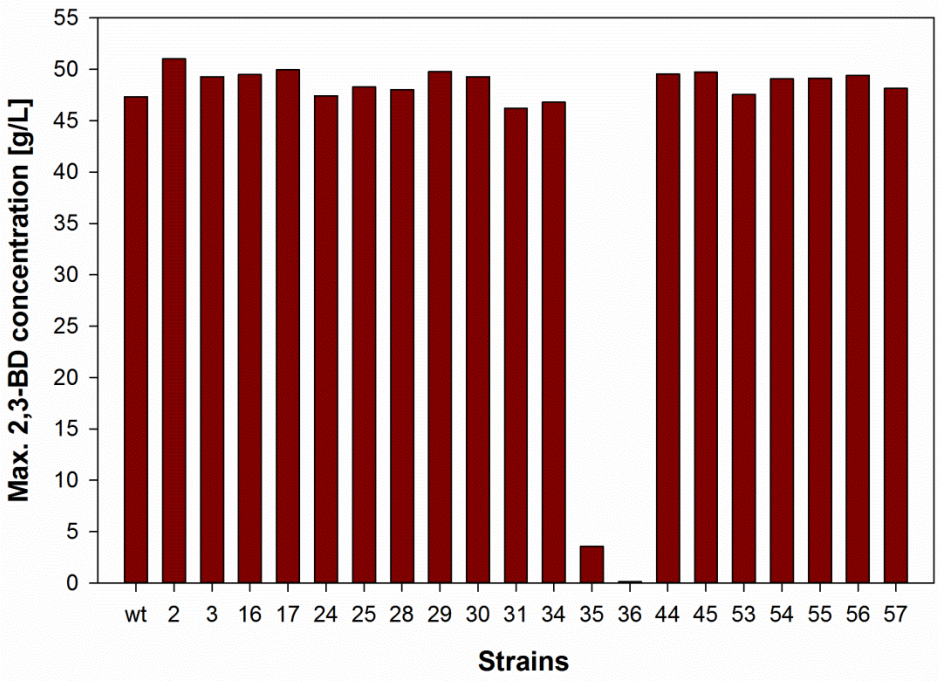


Figure 3-4 Maximum 2,3-BD production by potential UV-mutants of *B. licheniformis* DSM 8785. Conditions: 500 mL shake flasks, 100 mL medium (120 g/L glucose), 30°C, 100 rpm, 36-48 h. wt = wild type strain *B. licheniformis* DSM 8785 [joint work with Zhou, 2012]

2,3-BD production of the selected mutants was compared to the wild type strain. For the wild type strain the maximum amount of 2,3-BD (47.3 g/L) was reached after 36 h of cultivation. Among the selected potential mutants, strain no. 2 showed the highest 2,3-BD concentration of 51 g/L. For most tested mutants, maximum product concentrations ranged between 46.2 and 51 g/L, with the exception of strains no. 35 and 36, where 2,3-BD amounts were below 5 g/L. The increase in 2,3-BD production by random mutagenesis was 1.1% for the investigated potential mutants. However, maximum product concentrations were reached after 40-48 h for the mutant strains, leading to lower productivities.

Further experiments presented in this thesis with *B. licheniformis* DSM 8785 were performed using the wild type strain.

3.1.3 Isolation of microorganisms from natural habitats

In addition to the microorganisms purchased from culture collections, provided by project partners and obtained after random mutagenesis from wild type strains, self-isolated strains from environmental samples were also investigated.

The environmental samples (water, driftwood and addled wood) were collected from the Steinhuder Sea in Lower Saxony. After transfer of the samples to selective agar plates and incubation for 4 d at 25°C, no colonies were obtained from the water and addled wood. From the driftwood sample 12 colonies were isolated. The isolated strains were cultivated on medium containing 25 g/L glucose and 2,3-BD production was analyzed by TLC (Fig. 3-5). Results showed that after 40 h of cultivation, the strains no. 1, 2 and 5-8 produced 2,3-BD, while no production was detected for strains no. 3, 4 and 9-12.

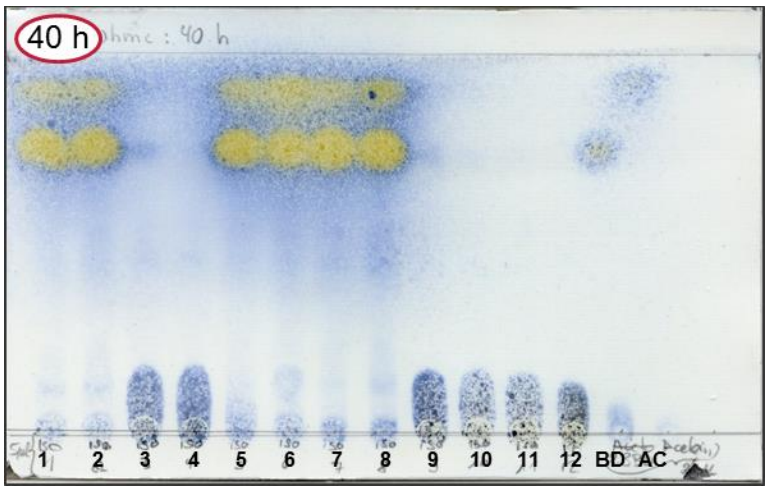


Figure 3-5 TLC of 2,3-BD production by isolated strains from driftwood (Steinhuder Sea, Lower Saxony); 5 µL samples (incl. 2,3-BD and Acetoin standards) [joint work with Iphöfer, 2011]

2,3-BD producing isolated strains (no. 1, 2 and 5-8) were identified by partial 16S rDNA-sequencing. The results of the strain identification are listed in Tab. 3-3.

Table 3-3 Results of partial 16S rDNA-sequencing

Strain No.	Results of partial 16S rDNA-sequencing	Risk group
1	*Enterobacter asburiae* *	2
2; 8	*Rhodococcus qingshengii* *	1
5; 6	*Enterobacter ludwigii* *	2
7	*Pseudomonas teessidea* *	2

* sequence identity ≥ 99.8 %

After identification, all 2,3-BD producing strains were cultivated in shake flasks on natural wood hydrolysate medium (containing 25 g/L glucose). 2,3-BD production by the isolated strains was compared to the wild type strains of *P. polymyxa* ATCC 12321 and *B. licheniformis* DSM 8785. Results from these cultivations are summarized in Tab. 3-4.

Table 3-4 Comparison of maximum 2,3-BD production, yield and productivity during cultivation of selected strains on natural wood hydrolysate medium (25 g/L glucose). Conditions: 500 mL shake flasks, 100 mL medium, 30°C, 100 rpm, 13-106h [joint work with Iphöfer, 2011]

Strain No.	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]
1*	**9.30**	**0.37**	0.13
2	**9.52**	**0.38**	0.13
5*	**9.52**	**0.38**	0.13
6*	7.94	0.32	0.17
7*	2.11	0.08	0.02
8	7.39	0.30	0.10
P. polymyxa ATCC 12321	**11.88**	**0.48**	**0.94**
B. licheniformis DSM 8785	**8.78**	**0.35**	**0.69**

Comparing maximum 2,3-BD concentrations and yields obtained during shake flask cultivations on wood hydrolysate medium, strains no. 1, 2 and 5 show good results, in the same range as *P. polymyxa* ATCC 12321 and *B. licheniformis* DSM 8785. The productivity for these strains is however only 0.13 g/(L*h), while the wild type strains reached values of 0.94 and 0.69 g/(L*h), respectively. The isolated strains were therefore not considered for further investigation.

3.2 2,3-BD production with *Paenibacillus polymyxa* ATCC 12321

After suitable non-pathogenic strains were selected, the process for 2,3-BD production was subjected to several optimization steps. Initial optimizations were performed on medium containing pure sugars as carbon source in the shake flask scale. The optimized parameters were then applied to cultivations on natural wood hydrolysates and to the bioreactor scale, where further optimization steps followed.

3.2.1 Shake flask experiments on glucose

In the shake flask scale, first optimizations were conducted concerning medium composition and cultivation parameters. Optimum values for the initial glucose concentration, the nature of the nitrogen source, shaking rate and incubation temperature were determined during batch cultivations with free and immobilized cells. Further investigations were performed using the fed-batch cultivation mode in order to increase 2,3-BD production.

3.2.1.1 DoE-Optimization of initial glucose concentration, shaking rate and temperature

The first step in the optimization of 2,3-BD production using *P. polymyxa* ATCC 12321 was a parameter optimization using experimental design (Design of Experiments, DoE). The design type employed was the Central Composite Design (CCD) and the software used for data interpretation was Minitab® 15. The CCD combines a full factorial design 2^k ("cube") with additional experiments, designated as "star" and "center point" in order to find optimal levels for the design variables (k) and aiming to achieve a nearly full spherical experimental area. For *P. polymyxa* ATCC 12321 three design variables (k = 3) were selected: the initial glucose concentration [g/L], the shaking rate [rpm] and the incubation temperature [°C] (Fig. 3-6).

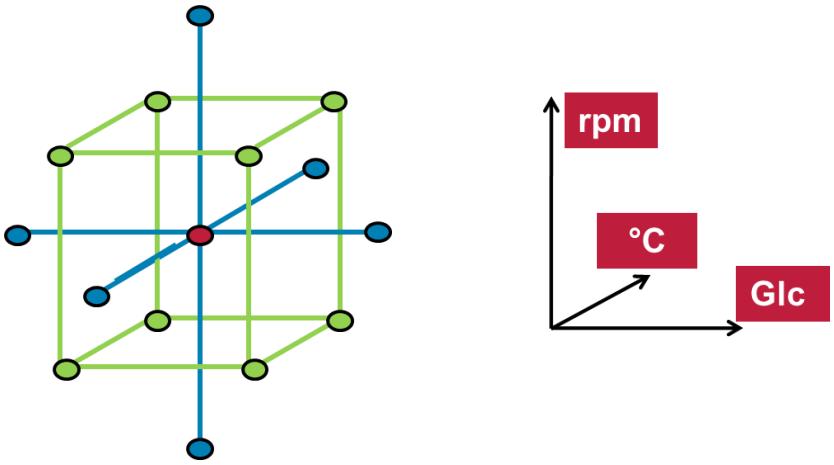


Figure 3-6 CCD experimental design for 3 variables selected for parameter optimization using *P. polymyxa* ATCC 12321 (modified after Kleppmann 2006)

Before the optimization of the selected variables could be performed, a reasonable range for these parameters ("study area") had to be determined. For the temperature, the lowest value was set at 25°C, since no cultivations at lower temperatures were found in data from literature. The maximum value was set to 35°C, considering the increased evaporation which accompanies cultivations at higher temperatures. For the shaking rate, the maximum value chosen was 150 rpm; higher shaking rates are not suitable for cultivations in 500 mL shake flasks with 2 baffles and 100 mL working volume, because the liquid reaches the cotton plug, increasing the risk for contamination. The lowest value for the shaking rate was set at 50 rpm considering that future bioreactor cultivations without stirring are not reasonable. Concerning the glucose concentration, preliminary cultivation tests were performed starting on medium with 20 g/L glucose and increasing the glucose concentration in 20 g/L steps. Fig. 3-7 shows the maximum 2,3-BD and residual glucose concentrations for the cultivations on medium with 20-80 g/L glucose.

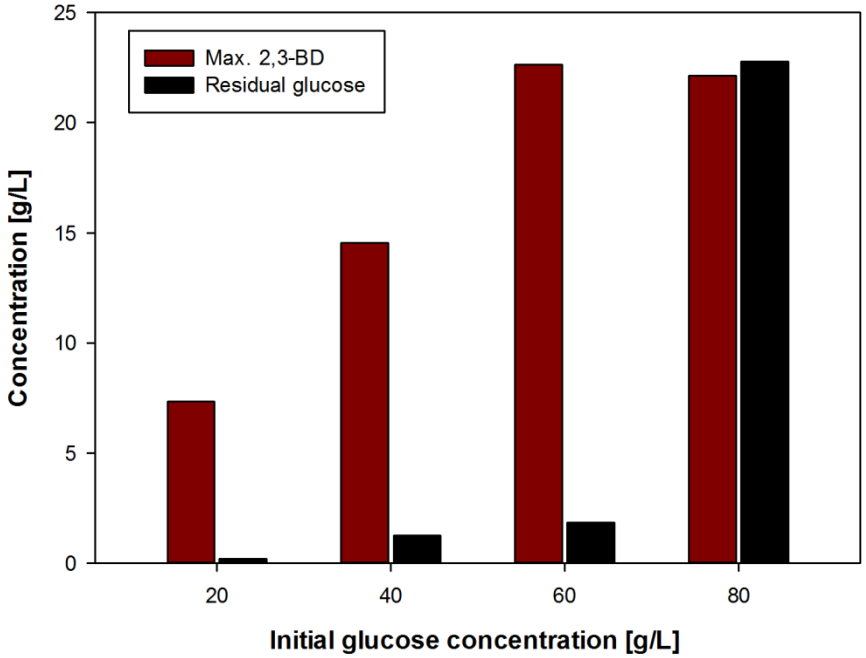


Figure 3-7 Maximum 2,3-BD production and residual glucose for cultivations using *P. polymyxa* ATCC 12321 on medium with 20-80 g/L glucose. Conditions: 500 mL shake flasks, 100 mL medium, 25 °C, 100 rpm and initial pH 6.6 not adjusted, 12-44 h [joint work with Iphöfer, 2011]

The highest product amount (22.6 g/L) was obtained from 60 g/L glucose with residual glucose concentrations below 2 g/L. An increase in initial glucose concentration to 80 g/L led to a similar amount of 2,3-BD (22.1 g/L), but significantly higher levels of residual glucose (22.8 g/L). According to these results, the study area for CCD optimization was determined (Tab. 3-5). Using the Minitab® 15 software, optimum values for the investigated parameters were determined using as response value maximum 2,3-BD production (Tab. 3-6, left) and productivity (Tab. 3-6, right).

Table 3-5 Study area for CCD optimization on glucose medium [joint work with Iphöfer, 2011]

Parameter	Level -1	Level 0	Level +1
Initial glucose [g/L]	50	70	90
Temperature [°C]	25	30	35
Shaking rate [rpm]	50	100	150

Table 3-6 Computed optimum parameters for *P. polymyxa* ATCC 12321 concerning maximum 2,3-BD production (left) and maximum productivity (right) [joint work with Iphöfer, 2011]

Parameters	Optimum values for maximum	
	2,3-BD production	productivity
Initial glucose [g/L]	68	50
Temperature [°C]	25	35
Shaking rate [rpm]	80	150

Maximum 2,3-BD production using the corresponding parameters was computed, yielding 23.4 g/L 2,3-BD. When performing the cultivation under the optimum parameters, 24.35 g/L 2,3-BD were obtained (see Appendix, Fig. 9-3). The values for maximum productivity were 1.4 g/(L*h) (computed) and 1.5 g/(L*h) (measured during cultivation; see appendix, Fig. 9-4).

Fig. 3-8 illustrates the surface plot for the chosen study area for obtaining maximum 2,3-BD amounts. The temperature was kept constant at 25°C.

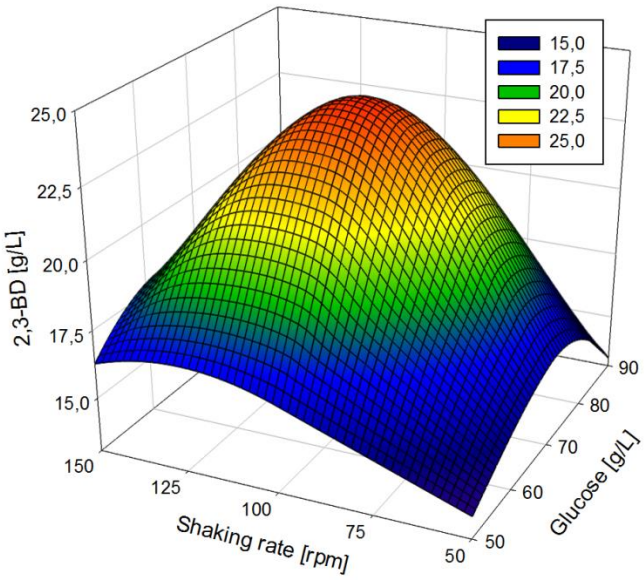


Figure 3-8 Surface plot of the chosen experimental area for maximum 2,3-BD production using *P. polymyxa* ATCC 12321 in the shake flask scale at 25°C [joint work with Iphöfer, 2011]

3.2.1.2 Comparison between the use of yeast extract / tryptone and urea as N-source

Due to the high price of complex medium components (e.g. yeast extract, tryptone), alternative nitrogen sources suitable for 2,3-BD production were investigated. A good candidate mentioned in literature is urea; its suitability as substitute for yeast extract and tryptone was tested during shake flask experiments on glucose. The amount of urea applied to the culture medium was calculated on the basis of the nitrogen content. Considering this approach, 2.73 g/L urea was determined as being equivalent to 5 g/L yeast extract and 5 g/L tryptone.

Fig. 3-9 illustrates a comparison between the cultivation of *P. polymyxa* ATCC 12321 on medium with 30 g/L glucose and yeast extract / tryptone (left) and the corresponding cultivation using urea as nitrogen source (right). During the cultivation with complex medium components, the entire amount glucose present in the culture medium was consumed after 30 h. At the same time, a maximum 2,3-BD concentration of 12 g/L could be observed. The cultivation using the alternative nitrogen source urea showed a reduced production of 2,3-BD (below 1 g/L), corresponding to a reduced consumption of glucose.

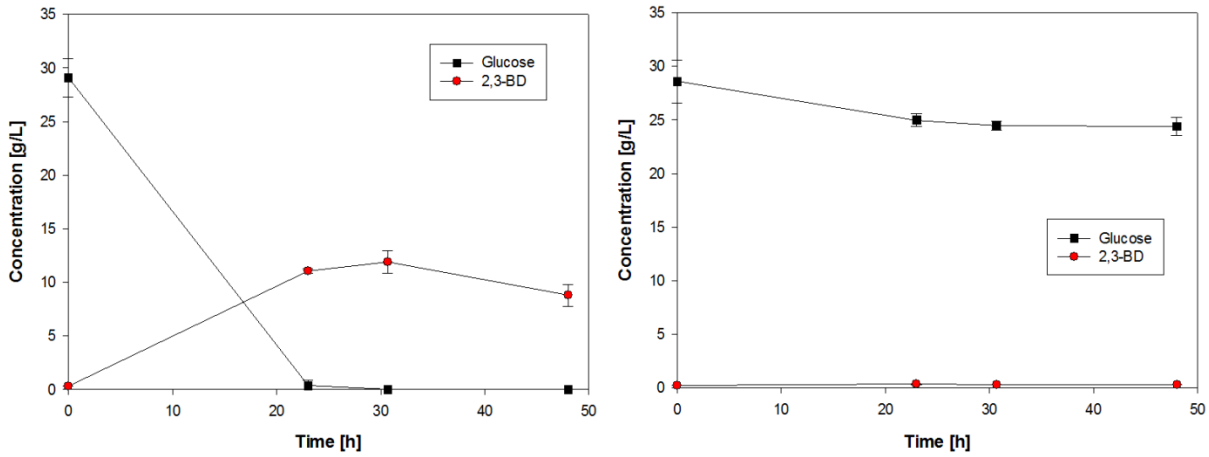


Figure 3-9 Comparison of the cultivation of *P. polymyxa* ATCC 12321 using yeast extract / tryptone (5 g/L each; left) and urea (2.73 g/L; right). Conditions: 500 mL shake flasks, 100 mL medium (30 g/L glucose), 30 °C, 100 rpm, initial pH 6.6 (not adjusted) [joint work with Iphöfer, 2011]

3.2.1.3 Cultivations using immobilized cells (LentiKats®)

Apart from the cultivations performed with free cells of *P. polymyxa* ATCC 12321, additional experiments were conducted using immobilized cells in form of LentiKats®. The immobilization procedure was performed by our project partners from the Institute of Agricultural Technology of the Thünen-Institute.

Fig. 3-10 shows the time course for a repeated-batch cultivation with immobilized cells of *P. polymyxa* ATCC 12321. 20 g wet LentiKats® (3.5-4 mm) were added to 100 mL medium (with 68 g/L glucose) and the cultures were incubated at 25°C and 80 rpm. The amount of cell dry weight entrapped in the 20 g LentiKats® corresponded to a concentration of 0.04 g/L CDW, which is 2.5 times higher compared to the initial CDW used for inoculation in the case of cultivations using free cells. The experiments consisted of three batch cultivation phases and two medium replacements (with identical initial sugar concentration) were carried out. The LentiKats® were separated from the culture broth, washed twice with sterile 9 g/L NaCl solution, then added to the Erlenmeyer flask containing 100 mL fresh medium with 68 g/L glucose and incubated under the same conditions mentioned above.

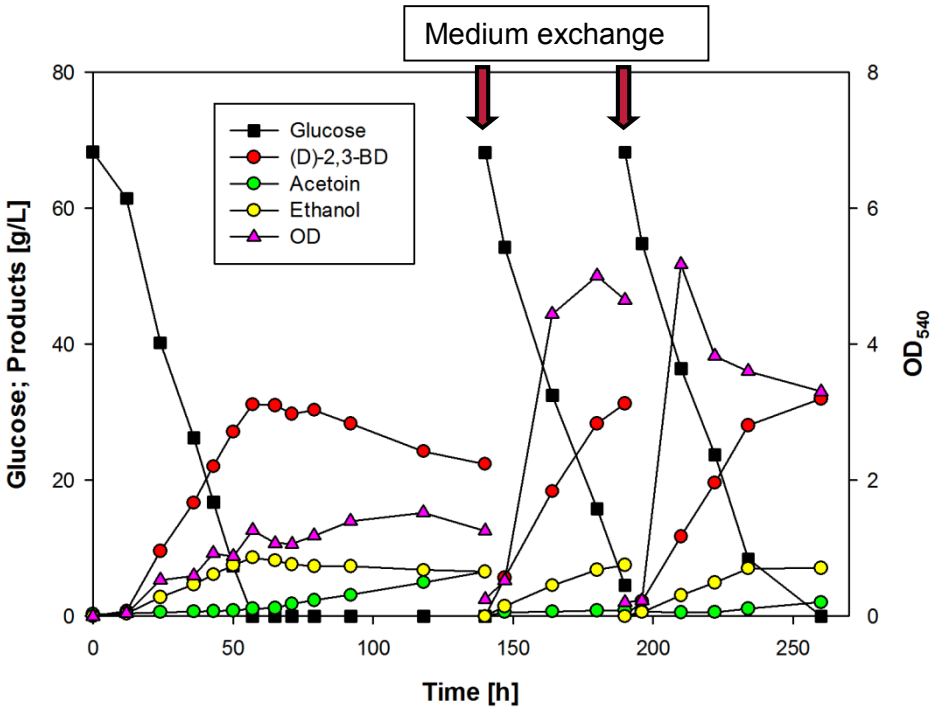


Figure 3-10 Time course for the repeated-batch cultivation with immobilized *P. polymyxa* ATCC 12321 (LentiKats®). Conditions: 500 mL shake flasks, 100 mL medium (68 g/L glucose), 25 °C, 80 rpm, initial pH 6.6 (not adjusted); time points for medium exchange are indicated by arrows [joint work with Iphöfer, 2011]

The results showed a maximum 2,3-BD production of approx. 31 g/L, 50-60 h after cultivation start and/or medium replacement. The main by-products reached amounts below 2 g/L for acetoin and between 6-8 g/L for ethanol.

3.2.1.4 Fed-batch cultivations

The optimum glucose concentration for maximum 2,3-BD production using *P. polymyxa* ATCC 12321 was identified at 68 g/L. In this case, the highest product amount (24.35 g/L) was detected after 64.5 h (see chapter 3.2.1.1). Since higher initial glucose concentrations could not be applied in order to increase 2,3-BD production, the fed-batch cultivation mode was employed. Due to the low productivity of 0.38 g/(L*h) obtained during the batch cultivation under above mentioned conditions, the optimized parameters for maximum productivity (50 g/L glucose, 35°C, 150 rpm) were considered instead. The batch cultivation under these conditions led to a maximum amount of 15.47 g/L 2,3-BD after 10 h, corresponding to a productivity of 1.5 g/(L*h).

Fig. 3-11 shows the time course for a fed-batch cultivation of *P. polymyxa* ATCC 12321. The cultivation was started with a glucose concentration of 50 g/L and incubated at 35°C and 150 rpm. After 10 h, before the entire amount of glucose was consumed, 5 g glucose and corresponding amounts of the other medium components were added in solid form to the culture. The working volume was adjusted to 100 mL after each sampling by addition of sterile Milli-Q water and the culture was incubated under the same conditions as before. Further two feeding steps were performed after 20 h and 30 h of cultivation.

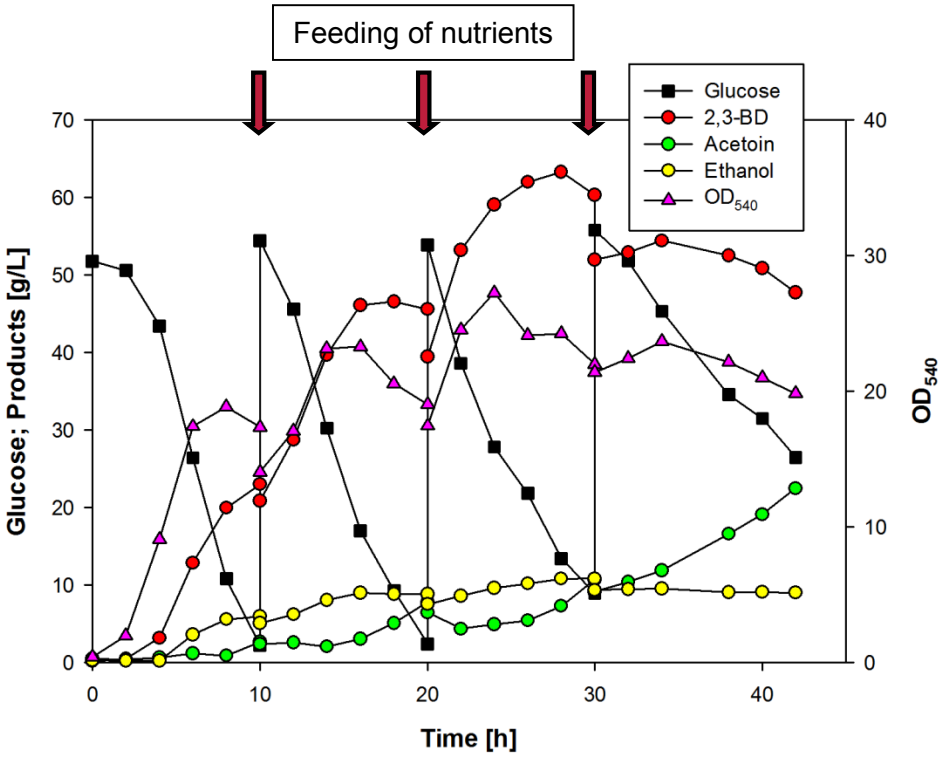


Figure 3-11 Time course for the fed-batch cultivation with *P. polymyxa* ATCC 12321. Conditions: 500 mL shake flasks, 100 mL medium (50 g/L glucose), 35 °C, 150 rpm, initial pH 6.6 (not adjusted); feeding time points are indicated by arrows [joint work with Iphöfer, 2011]

The results showed that using the fed-batch cultivation mode, maximum 2,3-BD production could be enhanced up to 63.3 g/L after 28 h of cultivation. Consequently, an increase in maximum productivity up to 2.26 g/(L*h) could be noted. The amounts of by-products measured at the time point of maximum 2,3-BD production were 7.8 g/L acetoin and 10.8 g/L ethanol. Bacterial growth stopped after 26 h cultivation and the OD values continued decreasing after the third feeding step. Considering the fact that the highest 2,3-BD amount was reached before the third feeding was performed, 63.3 g/L 2,3-BD were obtained from a total amount of 150 g/L glucose. This corresponds to a yield of 0.42 g/g glucose.

3.2.1.5 Balance on shake flask cultivations on glucose

All results of shake flask cultivations on glucose are summarized in Tab. 3-7. For the batch cultivation mode, the highest production and yield were obtained using the CCD optimized conditions for maximum 2,3-BD concentration: 68 g/L glucose, 25°C and 80 rpm. Results achieved with free and immobilized cells were in the same range. However, higher values for 2,3-BD concentration, yield and productivity were obtained using immobilized cells. On the contrary, the highest productivity was reached during Batch experiment No. 2, using CCD optimized conditions for maximum productivity: 50 g/L glucose, 35°C and 150 rpm. The same conditions were employed for the fed-batch cultivation, leading to a significant enhancement of maximum 2,3-BD amount, yield and productivity.

Table 3-7 Comparison between cultivations using free / immobilized *P. polymyxa* ATCC 12321 and batch / fed-batch cultivation modes [joint work with Iphöfer, 2011]

Cultivation type	Time [h]	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]
Batch No. 1 *	64.5	24.35	0.36	0.38
Batch No. 2 **	10	15.47	0.33	1.55
LentiKats® * (2x medium exchange)	50-60	~ 31 (after each medium exchange)	0.46-0.47	0.52-0.66
Fed-batch ** (4x50 g/L glucose)	28	63.28	0.46	2.26

Cultivation conditions: * 68 g/L glucose; 5 g/L yeast extract; 5 g/L tryptone; 25°C; 80 rpm
** 50 g/L glucose; 5 g/L yeast extract; 5 g/L tryptone; 35°C; 150 rpm

3.2.2 Shake flask experiments on wood hydrolysates

Further investigations in the shake flask scale were conducted on wood hydrolysates. For the production of natural wood hydrolysates, many preliminary steps are required and the process is time consuming. Therefore, an artificial wood hydrolysate medium was constructed and employed for optimization purposes, after comparison to the corresponding natural hydrolysate. Furthermore, the effect of potential inhibitory compounds present in natural wood hydrolysates on bacterial growth and 2,3-BD production was examined.

3.2.2.1 Comparison between cultivations on natural and artificial wood hydrolysates

A natural wood hydrolysate obtained from the fiber fraction of poplar wood (containing 30 g/L glucose) was delivered by our project partner from the Institute of Wood Technology of the Thünen-Institute. The composition of the natural wood hydrolysate is given in Tab. 3-8.

Table 3-8 Identified components of natural wood hydrolysate (fibre fraction of poplar wood)

Compound	Concentration [g/L]
Glucose	29.98
Xylose	0.97
Mannose	0.3
Furfural	0.023
5-Hydroxymethylfurfural	0.019
4-Hydroxybenzoic acid	0.008

Based on the identified compounds, a corresponding artificial wood hydrolysate medium was constructed, aiming to simulate a similar environment as natural wood hydrolysate medium. The artificial medium was intended for further optimization experiments. Previous to these optimizations, parallel cultivations were performed on natural and artificial wood hydrolysate medium. A comparison of glucose consumption and 2,3-BD production during these experiments is illustrated in Fig. 3-12 and Tab. 3-9..

Glucose consumption and 2,3-D production were slower in the beginning of the cultivation on natural wood hydrolysate medium. For both media, the highest 2,3-BD concentration was reached after 22 h. 10.9 g/L 2,3-BD were obtained using artificial medium and 11.6 g/L 2,3-BD from natural wood hydrolysate medium. For the natural medium, the highest amount of 2,3-BD was obtained before complete consumption of glucose; 1 g/L glucose were present in the medium at the corresponding time point.

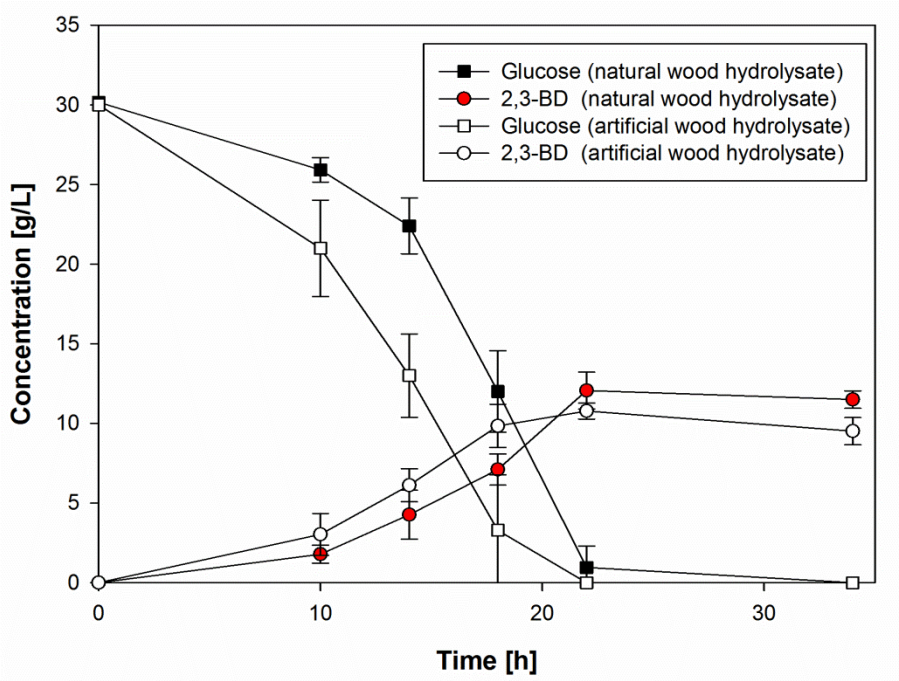


Figure 3-12 Comparison of *P. polymyxa* ATCC 12321 cultivation on natural and artificial wood hydrolysate medium. Conditions: 500 mL shake flasks, 100 mL medium (30 g/L glucose), 30 °C, 100 rpm, initial pH 6.6 (not adjusted) [joint work with Iphöfer, 2011]

Table 3-9 Comparison between cultivations using *P. polymyxa* ATCC 12321 on natural and artificial wood hydrolysate medium [joint work with Iphöfer, 2011]

Medium	Time [h]	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]
Natural wood hydrolysate	22	11.6	0.40	0.53
Artificial wood hydrolysate	22	10.9	0.36	0.50

The two types of media show similar results concerning maximum 2,3-BD production, yield and productivity. Due to this similar behavior, artificial wood hydrolysates can be used for further optimization studies.

3.2.2.2 Effect of potential inhibitory compounds from natural wood hydrolysates

Since natural wood hydrolysates contain substances, which may potentially inhibit bacterial growth, sugar consumption and 2,3-BD production, an important study was to investigate the influence of these compounds and to determine their corresponding inhibitory concentrations. The experiments were performed on artificial wood hydrolysate medium with previously determined optimum glucose concentration of 68 g/L.

The amounts for xylose (2.2 g/L) and mannose (0.6 g/L) were calculated according to data from natural wood hydrolysates and added to the artificial medium. The potential inhibitory compounds were tested separately or combined in various concentrations (1-fold, 2-fold, 4-fold, 8-fold and 16-fold). The 1-fold concentration corresponds to the concentration of these compounds in natural wood hydrolysate medium with 68 g/L glucose. The tested potential inhibitory compounds and the amounts are given in Tab. 3-10.

Table 3-10 Concentrations of potential inhibitory compounds in natural wood hydrolysates with 68 g/L glucose and 2-fold-16-fold amounts [joint work with Bieniek, 2011]

	Concentrations [g/L]					
Compound	**1-fold**	**2-fold**	**4-fold**	**8-fold**	**16-fold**	**32-fold**
Furfural	0.066	0.15	0.30	0.60	1.20	2.40
5-Hydroxymethylfurfural	0.089	0.20	0.40	0.80	1.60	3.20
4-Hydroxybenzoic acid	0.169	0.30	0.60	1.20	2.40	4.80
Vanillin	0.010	0.02	0.04	0.08	0.16	0.32
Syringaaldehyde	0.015	0.03	0.06	0.12	0.24	0.48
Formic acid	0.436	0.80	1.60	3.20	6.40	12.80
Acetic acid	1.526	3.00	6.00	12.00	24.00	48.00

The time course for glucose consumption and 2,3-BD production during cultivation on medium containing 68 g/L glucose and 0-4.8 g/L 4-hydroxybenzoic acid is illustrated in Fig. 3-13. During the cultivation without addition of 4-hydroxybenzoic acid, the entire glucose was consumed after 38 h of cultivation. The cultivation performed with the highest concentration of the inhibitor (4.8 g/L) showed no glucose consumption after 48 h. With increasing amount of 4-hydroxybenzoic acid, the glucose consumption in the culture medium was progressively slower. The same effect could be observed for 2,3-BD production: in the absence of the inhibitor, 26.5 g/L 2,3-BD were produced after 38 h; after 48 h in the presence of 4.8 g/L 4-hydroxybenzoic acid (32x concentration), no 2,3-BD production could be detected.

Fig. 3-14 shows the time course for glucose consumption and 2,3-BD production during cultivation on medium containing 68 g/L glucose and 0-24 g/L acetic acid. The cultivation without addition of acetic acid indicated a complete consumption of glucose after 48 h and a maximum amount of 25.8 g/L 2,3-BD after 43 h. During cultivations with addition of 1.526-6.0 g/L acetic acid, glucose consumption was faster (30-38 h). 2,3-BD production was about 10 h faster and maximum product amounts were in the range of 26.7-28.7 g/L. 12 g/L acetic acid led to an initially slower glucose consumption in the beginning, but also to an enhancement of maximum 2,3-BD production by 5 g/L, yielding 30 g/L 2,3-BD after 43 h. A complete inhibition

of both glucose consumption and product formation was noted during cultivation in the presence of 24 g/L acetic acid.

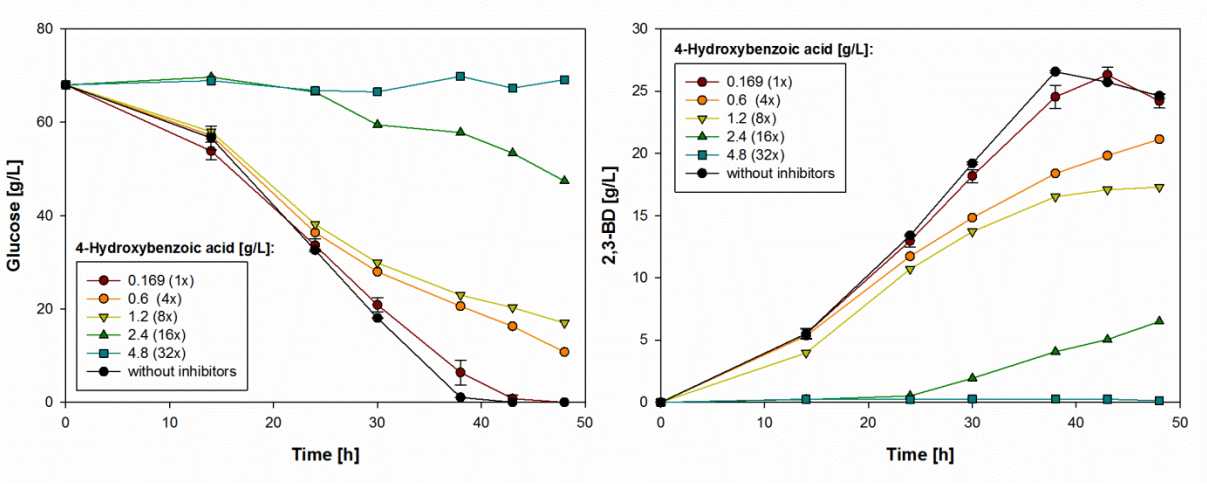


Figure 3-13 Glucose consumption (left) and 2,3-BD production (right) during cultivation of *P. polymyxa* ATCC 12321 on medium with 0-4.8 g/L 4-hydroxybenzoic acid. Conditions: 100 mL shake flasks, 20 mL medium (68 g/L glucose), 25°C, 80 rpm [joint work with Bieniek, 2011]

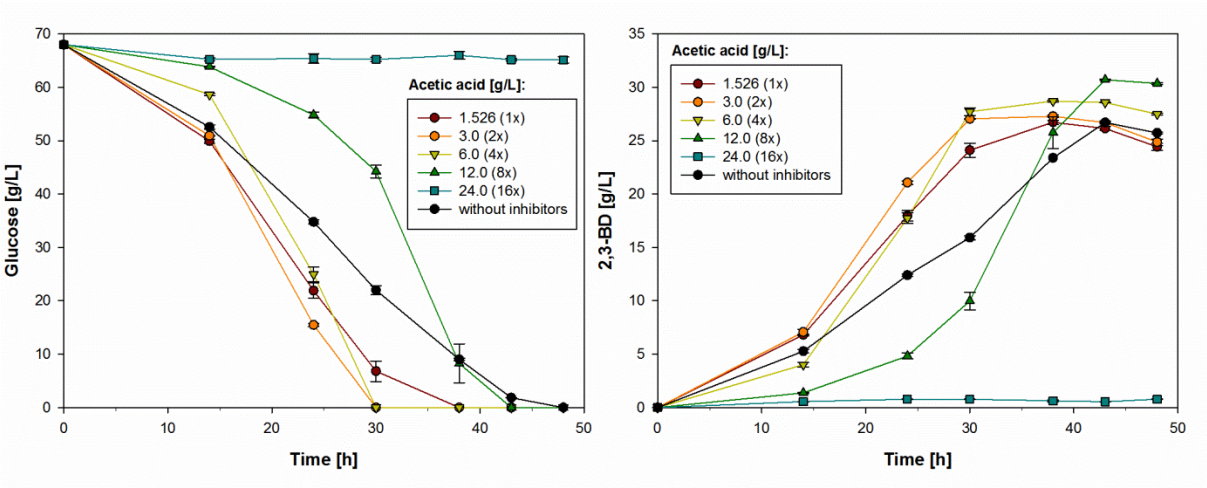


Figure 3-14 Glucose consumption (left) and 2,3-BD production (right) during cultivation of *P. polymyxa* ATCC 12321 on medium with 0-24 g/L acetic acid. Conditions: 100 mL shake flasks, 20 mL medium (68 g/L glucose), 25°C, 80 rpm [joint work with Bieniek, 2011]

The studies performed with addition of combined inhibitory compounds showed different results, depending on the amount of the added substances (Fig. 3-15). The entire glucose was consumed after 38 h in the presence of 1x-4x amount of inhibitors, while without inhibitors no residual glucose could be detected after 43 h. An increase in the amount of inhibitors (8x-16x concentrations) led to a complete inhibition of 2,3-BD production process with no glucose being consumed. The highest product yield was approx. 26.5 g/L 2,3-BD, obtained after 43 h without inhibitors and 38 h in the presence of 1x-4x compound concentrations.

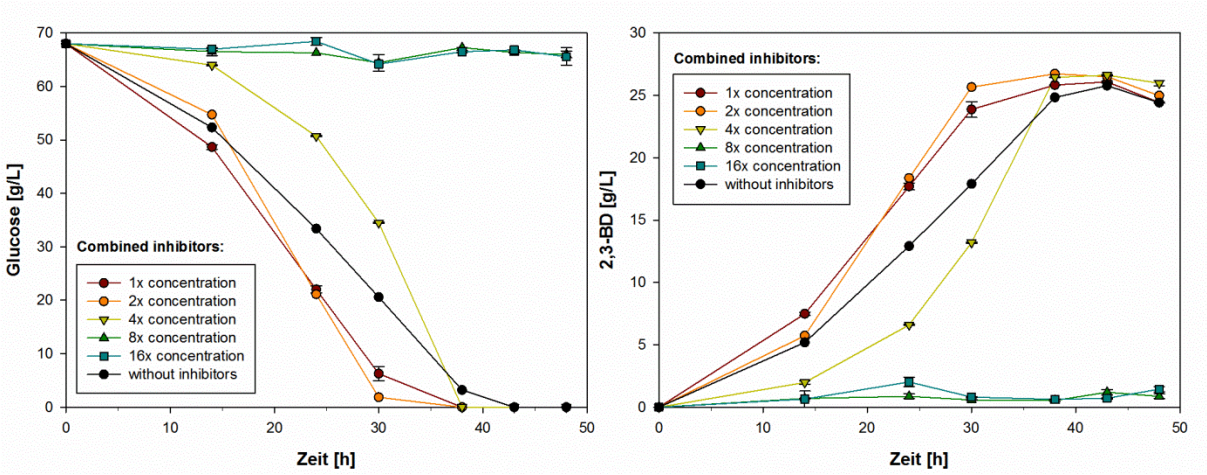


Figure 3-15 Glucose consumption (left) and 2,3-BD production (right) during cultivation of *P. polymyxa* ATCC 12321. Conditions: 100 mL shake flasks, 20 mL medium (68 g/L glucose and combined inhibitory compounds in 1x, 2x, 4x, 8x and 16x concentrations), 25°C, 80 rpm [joint work with Bieniek, 2011]

3.2.2.3 Balance on the effect of potential inhibitory compounds

The results of shake flask cultivations on artificial wood hydrolysate medium with addition of various amounts of potential inhibitory compounds are summarized in Tab. 3-11. No inhibiting effect could be detected during cultivation with compound amounts equivalent to those present in natural wood hydrolysates with 68 g/L glucose.

Table 3-11 Balance on the effect of potential inhibitory compounds in natural wood hydrolysates. No inhibition (-), inhibition (+), strong inhibition (++) [joint work with Bieniek, 2011]

	Inhibition at following concentrations:				
Compound	**1-fold**	**2-fold**	**4-fold**	**8-fold**	**16-fold**
Furfural*	-	-	-	-	-
5-Hydroxymethylfurfural**	-	-	-	-	-
4-Hydroxybenzoic acid	+	+	+	+	++
Vanillin***	-	-	-	-	-
Syringaaldehyde****	-	-	-	-	-
Formic acid	-	-	-	-	+
Acetic acid	-	-	-	-	++
Combined inhibitors	-	-	-	++	++

* inhibition from 32-fold amount (2.4 g/L)
** inhibition from 32-fold amount (3.2 g/L)
*** no inhibition until 32-fold amount (0.32 g/L)
**** inhibition from 32-fold amount (0.48 g/L)

3.2.3 Scale-up to the 3.5 L bioreactor on artificial wood hydrolysates

The optimized parameters from the shake flask scale were applied to cultivations in the bioreactor scale. The experiments in the 3.5 L bioreactor were carried out on artificial wood hydrolysate medium with previously determined optimum glucose concentration of 68 g/L. Besides glucose, the medium contained 2.2 g/L xylose and 0.6 g/L mannose. No inhibitory compounds were added to the culture medium. During bioreactor experiments, the main focus was optimizing the cultivation parameters in order to reproduce the results obtained in the shake flask scale.

3.2.3.1 Influence of the aeration rate on 2,3-BD production

The first bioreactor cultivation was conducted using the optimized conditions for maximum 2,3-BD production: 25°C and medium with 68 g/L glucose. For the stirring speed a value of 200 rpm and for the aeration rate 0.5 L/(L*min) were chosen according to data from literature (Nakashimada et al. 2000). According to the shake flask experiments, the pH was not controlled during cultivation. Fig. 3-16 shows the time course for the corresponding bioreactor cultivation. After 10 h, bacterial growth and glucose consumption started, which corresponded to a significant decrease in culture pH. A maximum OD_{540} value of 5.4 was measured after 26 h, when pH levels reached a minimum value of 5.0-5.2 and growth stopped. Glucose consumption was slow and after 70 h a residual amount of 45 g/L could be detected. Maximum 2,3-BD production reached 13.6 g/L after 44 h.

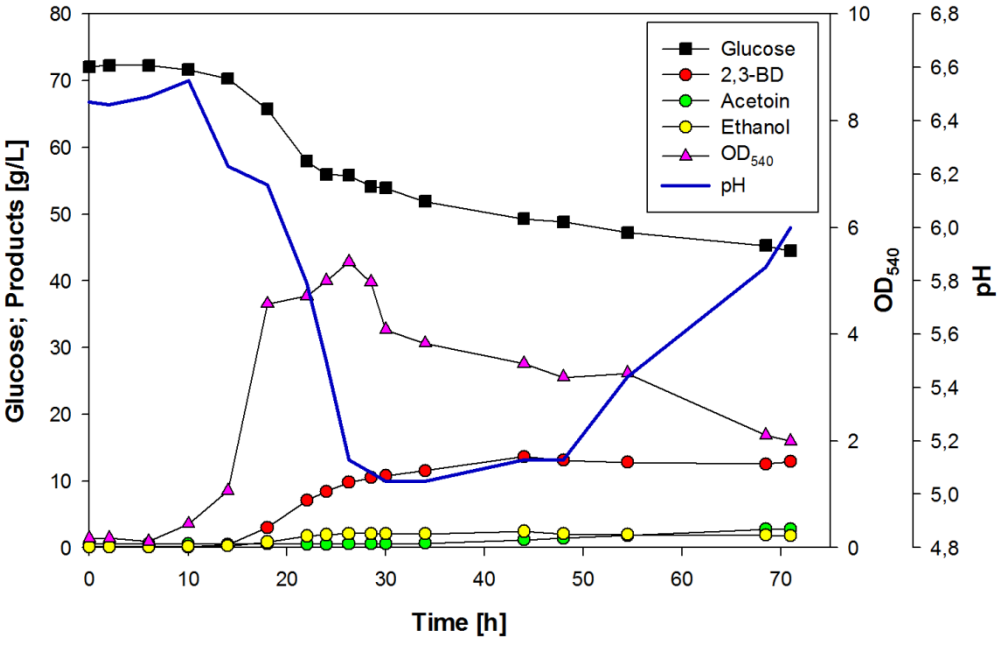


Figure 3-16 Time course for the batch cultivation of *P. polymyxa* ATCC 12321. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (68 g/L glucose), 200 mL preculture, 25°C, 200 rpm, aeration rate 0.5 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Iphöfer, 2011]

Due to the low bacterial growth and the reduced homogeneity of the cellular suspension, the stirring speed was increased to 400 rpm for the following cultivations. Furthermore, the aeration rate was increased from 0.5 to 0.8 L/(L*min) to ensure a better oxygen supply to the cells. The time course for the cultivation (Fig. 3-17) showed an increase of OD values to a maximum of 16.6 after 18 h, corresponding to 5.3 g/L cell dry weight. Culture pH decreased from initially 6.6 to 5.1. The complete amount of glucose was consumed after 56 h, when the highest concentration of 24.3 g/L 2,3-BD could be detected. 2,3-BD production was faster during the first 18 h. As main by-product, 10 g/L glycerol were produced in the first 20 h of cultivation and then consumed again. Acetoin production increased constantly during the entire cultivation to a value of 4.3 g/L. Further by-product concentrations were 3 g/L ethanol and 2.8 g/L lactate (results not shown).

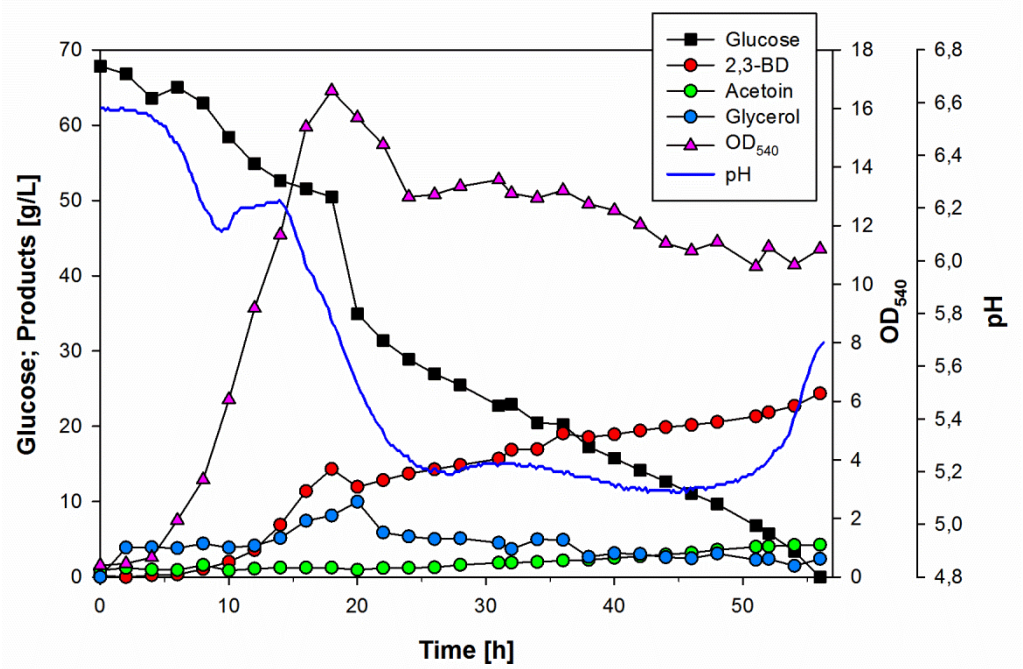


Figure 3-17 Time course for the batch cultivation of *P. polymyxa* ATCC 12321. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (68 g/L glucose), 200 mL preculture, 25°C, 400 rpm, aeration rate 0.8 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Bieniek, 2011]

A further increase of the aeration rate to 1.2 L/(L*min) (Fig. 3-18) led to a shorter cultivation time. After 48 h glucose consumption was complete and 25.9 g/L 2,3-BD was produced, corresponding to a production rate of 0.54 g/(L*h). The time course for the OD showed a steep increase in the first 20 h to a value of 17. Culture pH decreased from 6.6 to 5 and remained constant between 25-45 h. Acetoin production reached a maximum of 3 g/L before glucose consumption. The highest amount of glycerol was 9.4 g/L after 20 h and afterwards decreased slowly. Additionally, 3 g/L ethanol and 2 g/L lactate were obtained (results not shown).

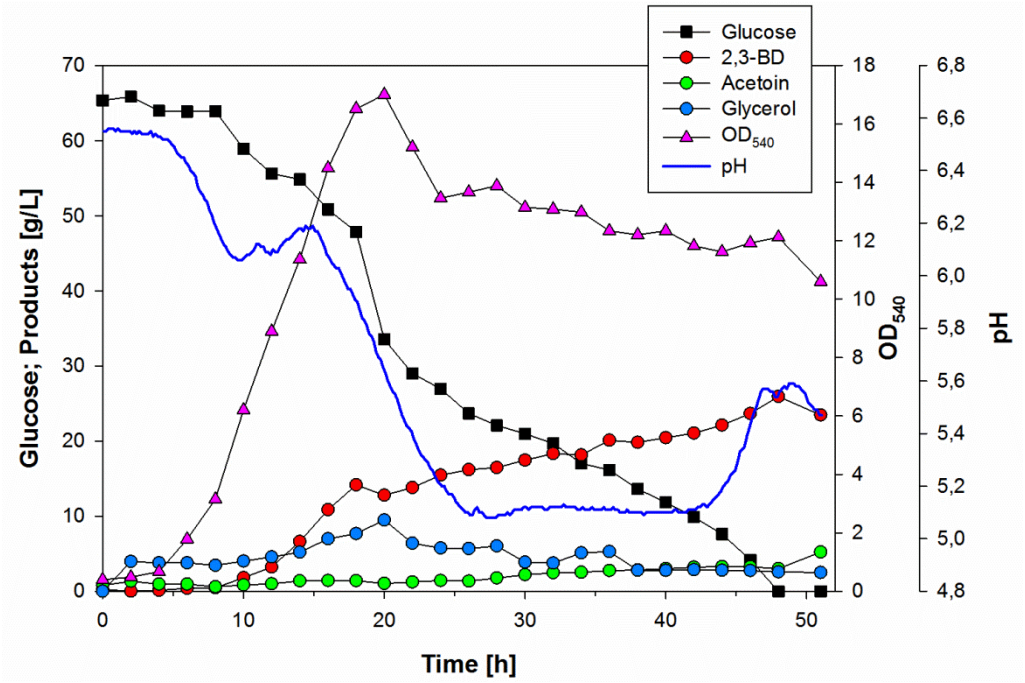


Figure 3-18 Time course for the batch cultivation of *P. polymyxa* ATCC 12321. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (68 g/L glucose), 200 mL preculture, 25°C, 400 rpm, aeration rate 1.2 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Bieniek, 2011]

A second bioreactor cultivation was performed using an aeration rate of 1.2 L/(L*min), but at a temperature of 30°C (Fig. 3-19). During this experiment, the maximum growth with an OD value of 12 was reached after 12 h. The glucose amount was completely consumed after only 38 h and a maximum 2,3-BD concentration of 25.3 g/L was obtained, with a production rate of 0.67 g/(L*h). Culture pH decreased from the initial value of 6.6 to approximately 5.1. Acetoin production reached 2.5 g/L before glucose depletion, while ethanol and lactate concentrations were 2.9 and 2.8 g/L, respectively (results not shown).

For bioreactor cultivations at 30°C, a higher aeration rate of 1.5 L/(L*min) was investigated as well. The time course for the cultivation is illustrated in Fig. 3-20. Maximum values for the OD (12.8) were obtained after 14 h. The time course for glucose consumption showed a faster decrease in sugar concentration between 8 and 16 h and slower between 16 and 36 h. A residual amount of 9 g/L glucose remained in the culture broth. The highest product concentration, 20.7 g/L 2,3-BD, was reached after 36 h. Acetoin production started after 16 h and an amount of 3.3 g/L was obtained at the time point of maximum 2,3-BD production. As by-products, 2.3 g/L lactate and 2.6 g/L ethanol (result not shown) were measured. Culture pH decreased from the initial value of 6.6 to 5.0 after 16 h and remained at the same level until 30 h, when it started increasing again.

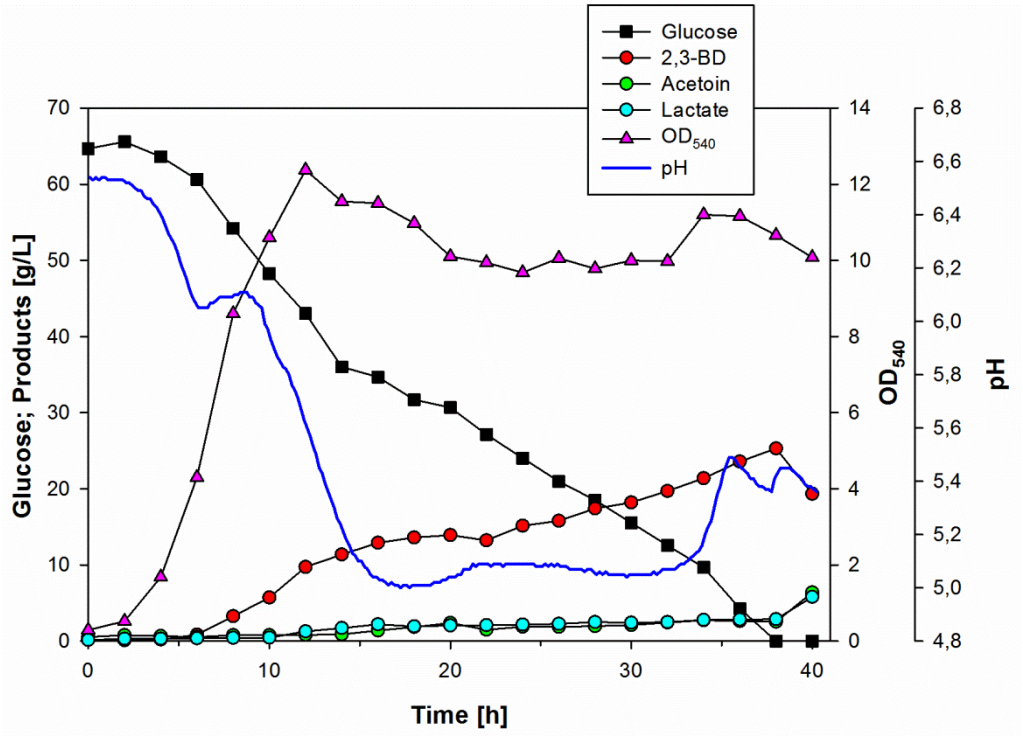


Figure 3-19 Time course for the batch cultivation of *P. polymyxa* ATCC 12321. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (68 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Bieniek, 2011]

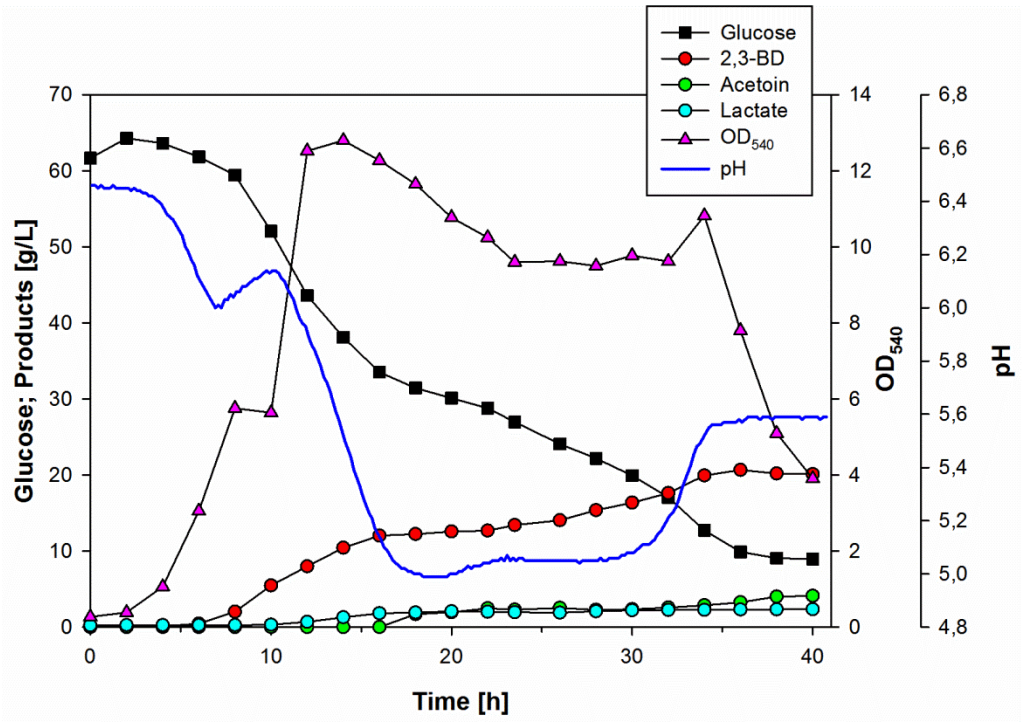


Figure 3-20 Time course for the batch cultivation of *P. polymyxa* ATCC 12321. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (68 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.5 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Bieniek, 2011]

3.2.3.2 Cultivations with forced pH-shifts

Petrov and Petrova (2010) were able to improve 2,3-BD production from glycerol with *Klebsiella pneumoniae* G31 using a new developed method of forced pH fluctuations. Based on the data from literature, the method of forced pH shifts was employed in this thesis for *P. polymyxa* ATCC 12321 in an attempt to increase 2,3-BD production. After the pH dropped to a certain level, 10% NaOH solution was added to reach a pH increase of 0.5 or 1 unit, respectively. Three different forced pH shifts from 5.8 → 6.3 (Fig. 3-21), 5.3 → 5.8 (Fig. 3-22) and 5.3 → 6.3 (Fig. 3-23) were tested at 30°C, 400 rpm and 1.2 L/(L*min) aeration rate.

The time course for the cultivation with forced pH shifts from **5.8 → 6.3** showed a maximum OD of 12 after 14 h. The glucose concentration decreased continuously until 32 h, when 23.6 g/L 2,3-BD were reached, corresponding to a production rate of 0.74 g/(L*h). The measured by-products were 6 g/L lactate, 2 g/L acetoin and 3.5 g/L ethanol (result not shown).

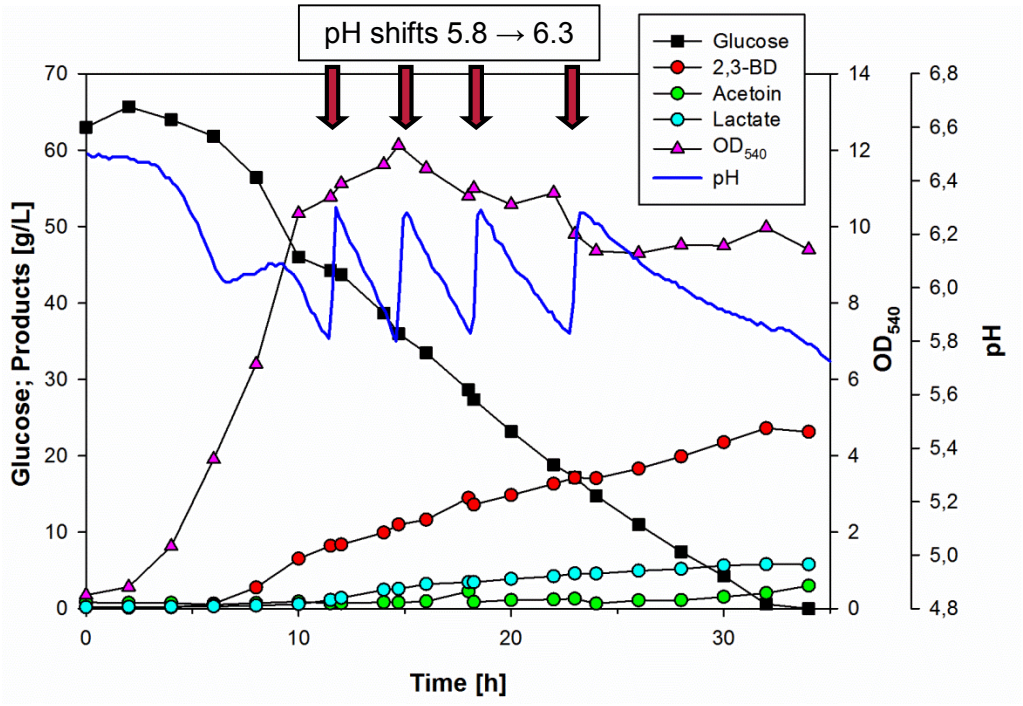


Figure 3-21 Time course for the batch cultivation of *P. polymyxa* ATCC 12321. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (68 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), pH-shifts (5.8 → 6.3) indicated by arrows [joint work with Bieniek, 2011]

With forced pH shifts from **5.3 → 5.8** maximum 2,3-BD production reached 25.7 g/L after 32 h, with a production rate of 0.80 g/(L*h), when glucose was completely consumed. 4 g/L lactate, 1.9 g/L acetoin and 3.1 g/L ethanol (result not shown) were obtained as by-products.

During cultivation with forced pH shifts from **5.3 → 6.3** glucose was consumed after 36 h and 22.2 g/L 2,3-BD were obtained, with a production rate of 0.62 g/(L*h). By-product formation showed amounts of 5.6 g/L lactate, 4.6 g/L acetoin and 2.8 g/L ethanol (result not shown).

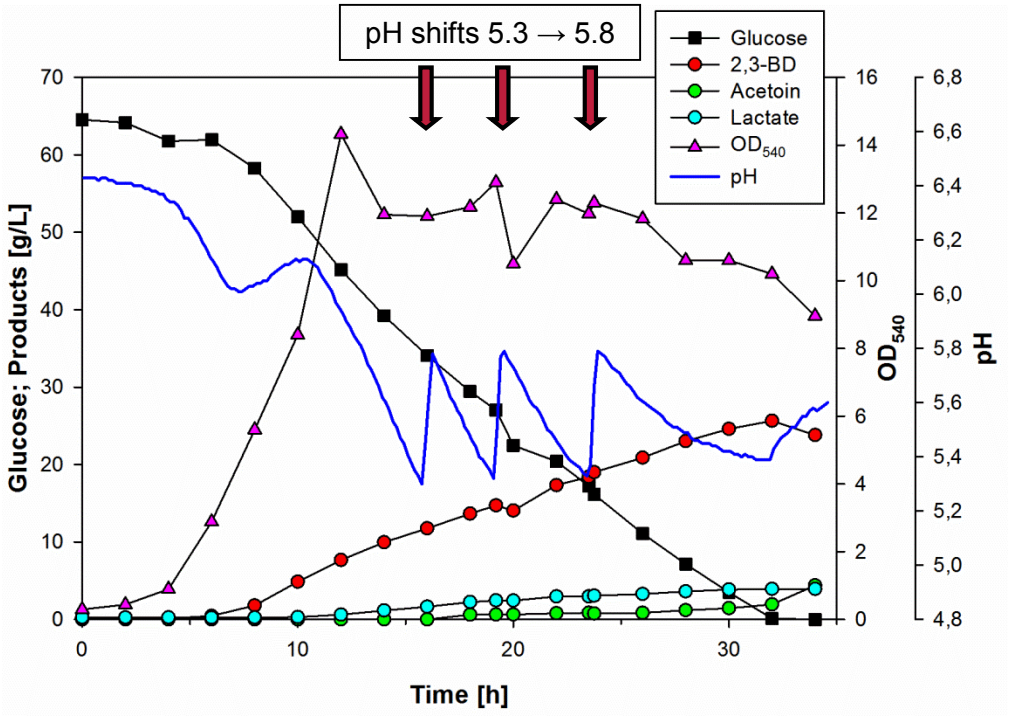


Figure 3-22 Time course for the batch cultivation of *P. polymyxa* ATCC 12321. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (68 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), pH-shifts (5.3 → 5.8) indicated by arrows [joint work with Bieniek, 2011]

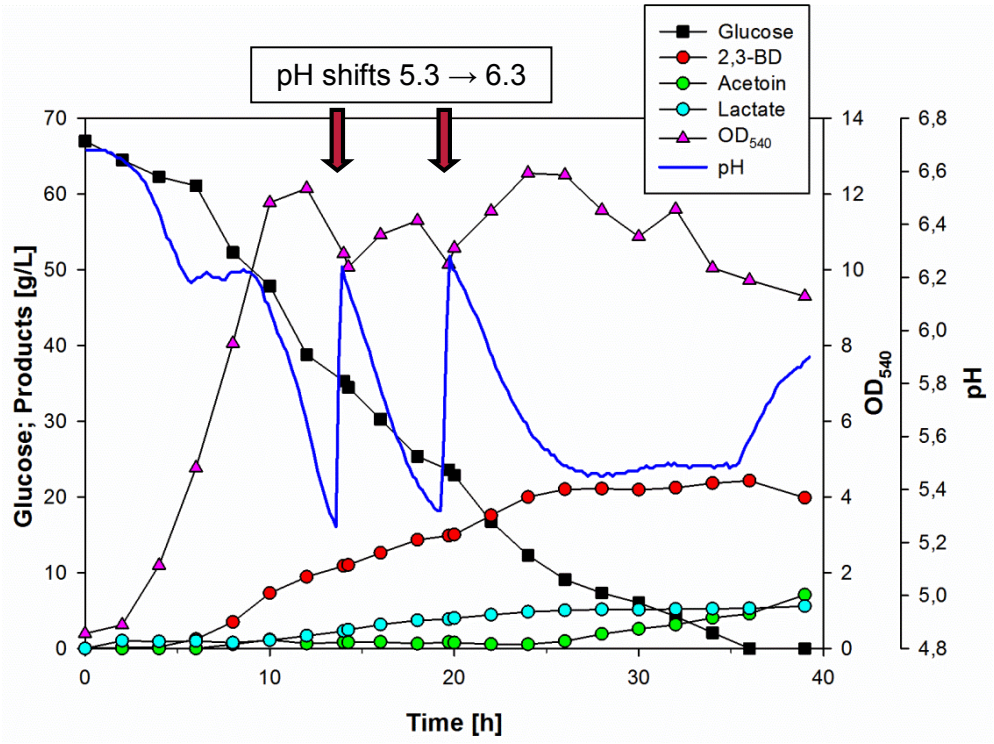


Figure 3-23 Time course for the batch cultivation of *P. polymyxa* ATCC 12321. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (68 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), pH-shifts (5.3 → 6.3) indicated by arrows [joint work with Bieniek, 2011]

3.2.3.3 Fed-batch cultivations

During batch experiments with *P. polymyxa* ATCC 12321 in the 3.5 L bioreactor scale, results obtained in the shake flask scale could be reproduced. An increase in 2,3-BD production was attempted by employing the fed-batch cultivation mode. The feedings were performed by adding the corresponding amount of nutrients in solid form to the culture, in order to avoid a dilution effect.

Fig. 3-24 shows the time course for a fed-batch cultivation of *P. polymyxa* ATCC 12321. The cultivation was started with a glucose concentration of 68 g/L and incubated at 30°C, 400 rpm and 1.2 L/(L*min). After 18 h and 54 h two feeding steps were performed; each time, 114 g glucose (38 g/L) and corresponding amounts of the other medium components were added. After 78 h additional 84 g glucose (28 g/L) and corresponding amounts of the other nutrients were supplemented to the culture broth. The OD showed a local maximum of 12 (15 h), increased to 15 (30 h) and further to 16.5 (72 h). 2,3-BD concentration increased after the first two feedings, reaching a maximum of 44.5 g/L after 78 h. Acetoin production started after 30 h, when 2,3-BD levels remained constant; after 54 h, 2,3-BD amounts increased again and acetoin concentration remained at about 8-10 g/L until the end of the cultivation. 6.5 g/L lactate and 2.9 g/L ethanol were also produced. The cultivation was performed without forced pH shifts; however, natural pH fluctuations could be observed with each feeding step.

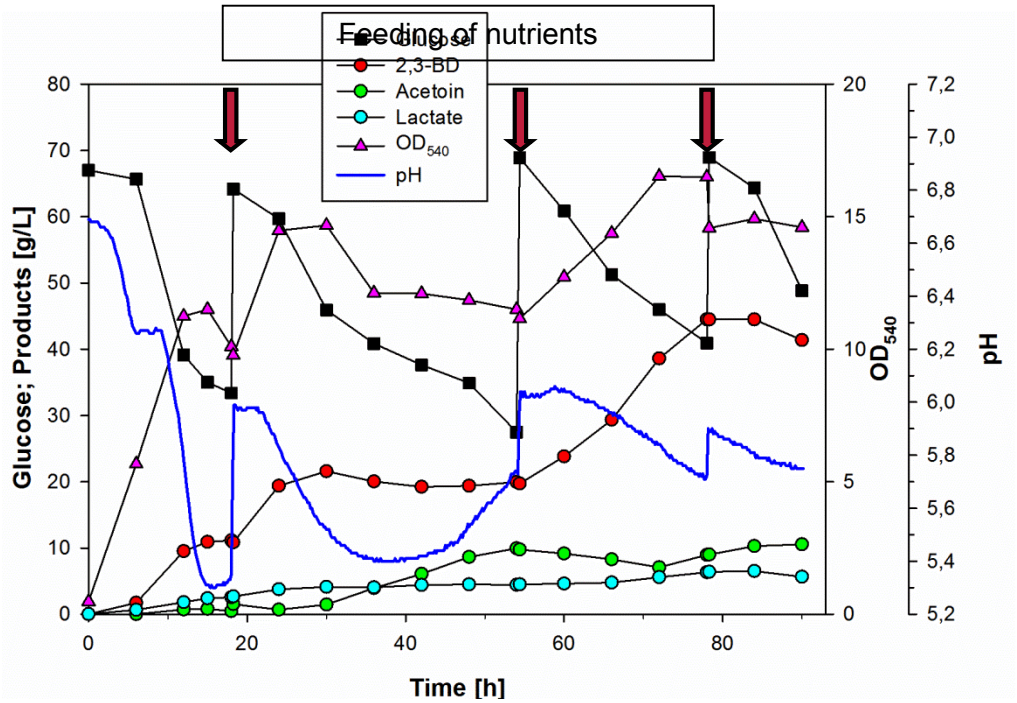


Figure 3-24 Time course for the fed-batch cultivation of *P. polymyxa* ATCC 12321. Conditions: 3.5 L bioreactor, 2.8 L artificial wood hydrolysate medium (68 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min); feeding time points are indicated by arrows [joint work with Bieniek, 2011]

3.2.3.4 Balance on cultivations in the 3.5 L bioreactor scale

Tab. 3-12 summarizes the results of the bioreactor cultivations with *P. polymyxa* ATCC 12321 on artificial wood hydrolysate medium. The results of the experiments at different aeration rates showed a maximum 2,3-BD production of 25.3 g/L and a yield of 0.38 for the cultivation performed at 25°C with an aeration rate of 1.2 L/(L*min). Increasing the temperature to 30°C led to similar results regarding 2,3-BD production and yield; a significant increase in productivity could be noted, corresponding to the shorter cultivation time. Optimum conditions for batch cultivation were 30°C, 400 rpm and 1.2 L/(L*min) aeration rate.

Employing the method of forced pH shifts led to a constant glucose consumption and 2,3-BD production rate throughout the entire cultivation process. Furthermore, the maximum 2,3-BD concentration was reached 6 h sooner for the optimum pH shift (5.3 → 5.8), leading to an enhanced productivity.

During the experiment performed using the fed-batch cultivation mode, 2,3-BD production and yield could be increased up to 44.5 g/L and 0.43 g/g glucose, respectively.

Table 3-12 Comparison between bioreactor cultivations with *P. polymyxa* ATCC 12321 on artificial wood hydrolysate medium (68 g/L glucose) under various conditions [joint work with Bieniek, 2011; Iphöfer, 2011]

Conditions	Time [h]	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]
25°C; 200 rpm; 0.5 L/(L*min)	44	13.6	0.35	0.31
25°C; 400 rpm; 0.8 L/(L*min)	56	24.4	0.36	0.44
25°C; 400 rpm; 1.2 L/(L*min)	48	**25.9**	**0.38**	0.54
30°C; 400 rpm; 1.2 L/(L*min)	38	25.3	0.37	**0.67**
30°C; 400 rpm; 1.5 L/(L*min)	36	20.7	0.36	0.57
30°C; 400 rpm; 1.2 L/(L*min)				
pH shift 5.8 → 6.3	32	23.6	0.35	0.74
pH shift 5.3 → 5.8	32	**25.7**	**0.38**	**0.80**
pH shift 5.3 → 6.3	36	22.2	0.33	0.62
Fed-Batch				
Glucose [g/L] 68+2x38+28	78	**44.5**	**0.43**	0.57

3.3 2,3-BD production with *Bacillus licheniformis* DSM 8785

During the screening process, the second strain identified as having a good potential for 2,3-BD production was *B. licheniformis* DSM 8785. Investigations regarding the optimization of medium composition and cultivation parameters were conducted in the shake flask scale using pure sugars. With optimized parameters, cultivations on wood hydrolysates were performed. Finally, a scale-up of 2,3-BD production process to the 3.5 L and 45 L bioreactor scale was carried out.

3.3.1 Shake flask experiments on pure sugars

Cultivation experiments using free cells of *B. licheniformis* DSM 8785 were performed using both batch and fed-batch cultivation modes. During batch cultivations, studies concerning the optimum initial sugar concentration for high 2,3-BD production were carried out. The nature and concentration of the nitrogen source as well as the incubation temperature were also investigated. The aim of the fed-batch cultivations was to start with the optimum initial glucose concentration and to reach maximum possible 2,3-BD concentrations by stepwise feeding of nutrients.

3.3.1.1 Influence of initial sugar concentration on 2,3-BD production

Batch cultivations in shake flasks were carried out using various initial glucose concentrations in order to detect the optimal conditions for 2,3-BD production with *B. licheniformis* DSM 8785. Fig. 3-25 shows a comparison of the maximum 2,3-BD concentrations reached with starting glucose amounts between 20 and 300 g/L as well as the time points, when these concentrations were reached. The results of these experiments were compared concerning 2,3-BD production, yield, productivity, residual glucose and acetoin formation (Tab. 3-13). With increasing initial glucose concentration, a significant increase in the produced amount of 2,3-BD could be observed. A maximum concentration of 72.6 g/L 2,3-BD was obtained after 86 hours of cultivation on medium with 180 g/L glucose. This corresponds to a 2,3-BD yield of 0.42 g/g glucose and a productivity of 0.86 g/(L*h). A further increase of the initial glucose concentration above 180 g/L led to a decrease in maximum 2,3-BD production as well as a significant increase in the residual sugar concentration. Furthermore, a high increase in acetoin (2,3-BD precursor) levels was observed for higher initial glucose concentrations. This was accompanied by a significant reduction in 2,3-BD yield and productivity. The highest 2,3-BD productivity, 0.99 g/(L*h), was reached with an initial glucose concentration of 120 g/L. Higher initial glucose concentrations led to a decrease in 2,3-BD productivity. 2,3-BD yield from glucose was in the same range (0.40-0.42 g/g) for initial glucose concentrations between 80 and 200 g/L.

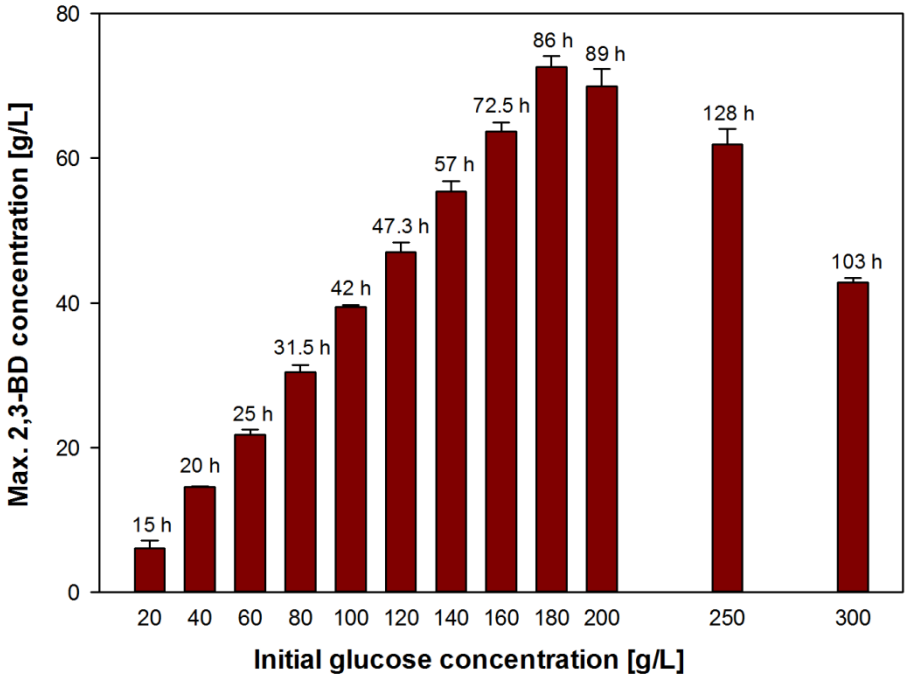


Figure 3-25 Effect of initial glucose concentration on 2,3-BD production with *B. licheniformis* DSM 8785. Conditions: 500 mL shake flasks, 100 mL medium containing 20-300 g/L glucose, 30 °C, 100 rpm and initial pH 6.6 not adjusted; time specifications above the bars indicate the time points of maximum 2,3-BD concentrations [joint work with Hamann, 2010; Zhou, 2012]

Table 3-13 Comparison of yield, productivity, residual glucose and acetoin concentrations during cultivation with *B. licheniformis* DSM 8785 using different initial glucose concentrations. Conditions: 500 mL shake flasks, 100 mL medium containing 20-300 g/L glucose, 30 °C, 100 rpm and initial pH 6.6 not adjusted [joint work with Hamann, 2010; Zhou, 2012]

Initial glucose [g/L]	2,3-BD yield [g/g]*	Productivity [g/(L*h)]	Residual glucose [g/L]	Acetoin [g/L]
20	0.30	0.40	0.0	1.59
40	0.36	0.73	0.0	2.02
60	0.38	0.87	2.43	1.19
80	0.41	0.96	5.53	1.54
100	0.40	0.94	1.74	4.53
120	0.40	**0.99**	2.45	3.46
140	0.40	0.97	1.95	3.74
160	0.40	0.88	1.15	3.99
180	**0.42**	0.86	7.03	5.39
200	0.40	0.79	26.43	7.19
250	0.31	0.48	47.90	21.49
300	0.25	0.42	129.89	15.22

* 2,3-BD yield is given in g/g consumed glucose

In addition to experiments on glucose, sucrose was also employed as sugar source for batch cultivations in the shake flask scale. Different initial sucrose concentrations in the range between 20-300 g/L were tested.

Fig. 3-26 shows a comparison of the maximum 2,3-BD concentrations obtained with initial amounts of sucrose between 20 and 300 g/L. The results of these experiments were compared concerning 2,3-BD production, yield, productivity and residual sucrose (Tab. 3-14). An increasing in 2,3-BD concentration was observed with increasing sucrose concentration in the culture medium. The maximum amount of 2,3-BD (77 g/L) was obtained from 260 g/L sucrose; the yield was 0.30 g/g and the productivity 0.75 g/(L*h). However, 11 g/L residual sucrose could be detected. The highest productivity was 1.04 g/(L*h), reached during the experiment with 180 g/L sucrose. The yield was in the same range for all experiments (0.28-0.32 g/g sucrose).

With a sucrose amount of 200 g/L, a good compromise between high 2,3-BD production (61.6 g/L) and productivity (0.99 g/(L*h)) could be obtained. Furthermore, residual sucrose concentration was below 3 g/L.

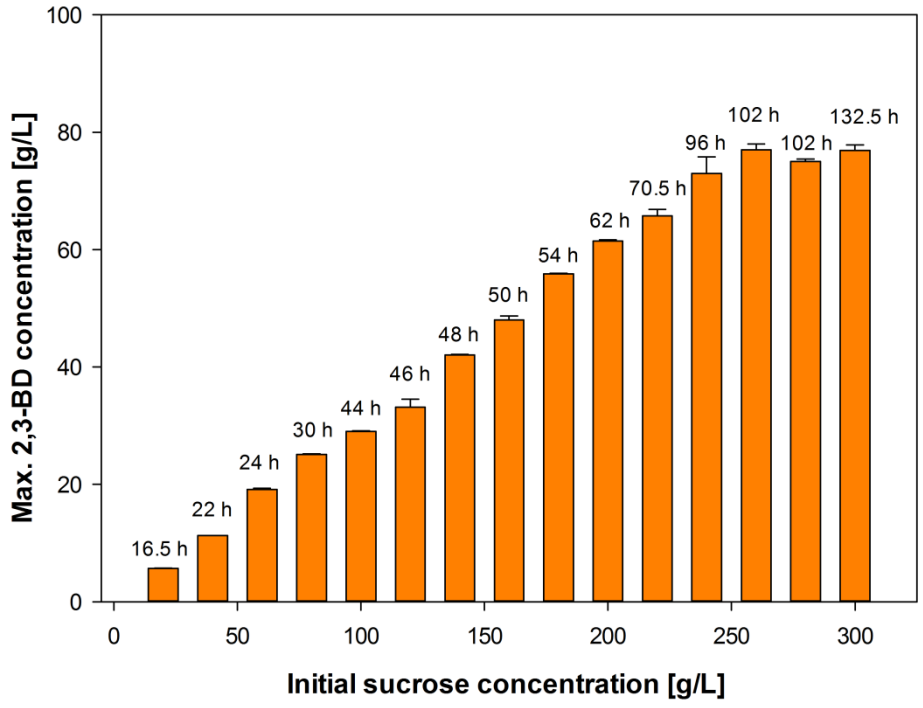


Figure 3-26 Effect of initial sucrose concentration on 2,3-BD production with *B. licheniformis* DSM 8785. Conditions: 500 mL shake flasks, 100 mL medium containing 20-300 g/L sucrose, 30 °C, 100 rpm and initial pH 6.6 not adjusted; time specifications above the bars indicate the time points of maximum 2,3-BD concentrations

Table 3-14 Comparison of yield, productivity and residual sucrose during cultivation with B. licheniformis DSM 8785 using different initial sucrose concentrations. Conditions: 500 mL shake flasks, 100 mL medium containing 20-300 g/L sucrose, 30 °C, 100 rpm and initial pH 6.6 (not adjusted)

Initial sucrose [g/L]	2,3-BD yield [g/g]*	Productivity [g/(L*h)]	Residual sucrose [g/L]	Acetoin [g/L]
20	0.35	0.42	0.0	0.52
40	0.28	0.51	0.0	1.85
60	0.32	0.80	0.0	1.05
80	0.31	0.84	0.0	1.68
100	0.31	0.71	0.0	2.80
120	0.29	0.74	3.19	10.77
140	0.31	0.88	3.04	8.95
160	0.32	1.00	2.09	11.26
180	0.32	1.04	3.13	2.92
200	0.31	0.99	2.77	3.67
220	0.32	0.94	10.91	8.42
240	0.32	0.76	12.32	10.40
260	0.31	0.76	10.85	10.91
280	0.30	0.74	29.34	10.56
300	0.28	0.58	22.41	19.19

* 2,3-BD yield is given in g/g consumed sucrose

3.3.1.2 Influence of the cultivation temperature on 2,3-BD production

Shake flask cultivations with 100 g/L glucose were performed at different temperatures (25, 30 and 35°C). The results of these experiments were compared regarding maximum 2,3-BD and acetoin concentration, yield and productivity (Tab. 3-15). 2,3-BD production and yield showed no considerable difference between cultivation experiments carried out at 30°C and 35°C, but the productivity was higher at 30°C. At 35°C a lower acetoin production could be observed. Cultivation experiments at 25°C resulted in low amounts of 2,3-BD as well as low values for yield and productivity.

Furthermore, using an initial glucose concentration of 180 g/L additional shake flask experiments were carried out at 37°C. At 30°C a maximum 2,3-BD concentration of 72.6 g/L, a yield of 0.42 g/g glucose and a productivity of 0.86 g/(L*h) were obtained (Fig. 3-25).

Table 3-15 2,3-BD production, yield, productivity and acetoin production during cultivation with *B. licheniformis* DSM 8785. Conditions: 500 mL shake flasks, 100 mL medium (100 g/L glucose), 25-35 °C, 100 rpm and initial pH 6.6 (not adjusted) [joint work with Hamann, 2010]

Temperature [°C]	Max. 2,3-BD [g/L]	2,3-BD yield [g/g]	Productivity [g/(L*h)]	Acetoin [g/L]
25	13.8	0.14	0.35	4.5
30	**39.5**	**0.40**	**0.97**	10.0
35	**40.1**	**0.40**	0.93	1.9

Fig. 3-27 shows the time course for the shake flask cultivation on medium with 180 g/L glucose at 37°C. Glucose consumption occurred using a constant rate and after 75 h no residual glucose remained in the culture broth. Bacterial growth was fast in the beginning of the cultivation (0-20 h) and maximum OD_{540} values around 15 were reached after 47 h. 2,3-BD production rate was constant throughout the entire cultivation and the highest concentration of 70.1 g/L 2,3-BD was measured after 74 h. This corresponds to a 2,3-BD yield of 0.39 g/g glucose and a productivity of 0.95 g/(L*h). Maximum 2,3-BD concentration and yield were lower compared to the cultivation at 30°C, but a higher productivity could be detected (see Fig. 3-25, Tab. 3-13). Regarding by-product formation during the cultivation at 37°C, 5 g/L acetoin and 21.5 g/L glycerol were detected at the time point of maximum 2,3-BD production.

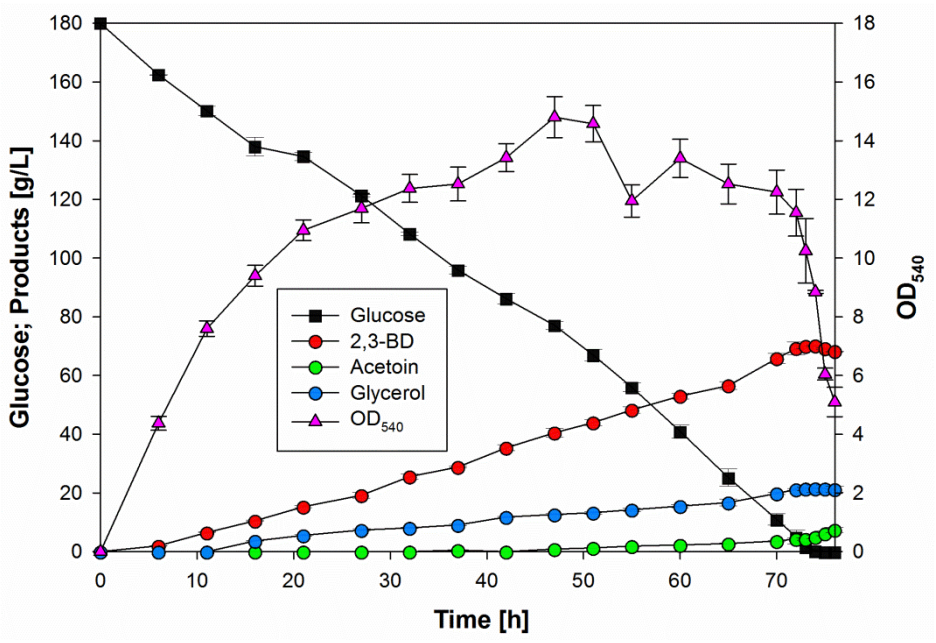


Figure 3-27 Time course of batch cultivation with *B. licheniformis* DSM 8785. Conditions: 500 mL shake flasks, 100 mL medium containing 180 g/L glucose, 37 °C, 100 rpm and initial pH 6.6 (not adjusted) [joint work with Ortmann, 2012]

3.3.1.3 Comparison between the use of yeast extract / tryptone and urea as N-source

As already investigated for *P. polymyxa* ATCC 12321 (chapter 3.2.1.2), a substitution of complex medium components (yeast extract, tryptone) by cheaper alternative nitrogen sources like urea was studied for *B. licheniformis* DSM 8785. Fig. 3-28 and Tab. 3-16 illustrate the results of the cultivations using *B. licheniformis* DSM 8785 on medium with 30 g/L glucose and yeast extract / tryptone and the corresponding cultivation using urea as nitrogen source. When employing complex medium components, glucose consumption was complete after 22.5 h and a maximum 2,3-BD concentration of 9.3 g/L could be measured. The cultivation using urea as alternative nitrogen source showed a maximum 2,3-BD production of 5.5 g/L after 26 h. Acetoin production started before glucose exhaustion, which occurred after 53 h. Acetoin levels increased to a maximum of 5.9 g/L. Furthermore, both yield and productivity were lower in the presence of urea.

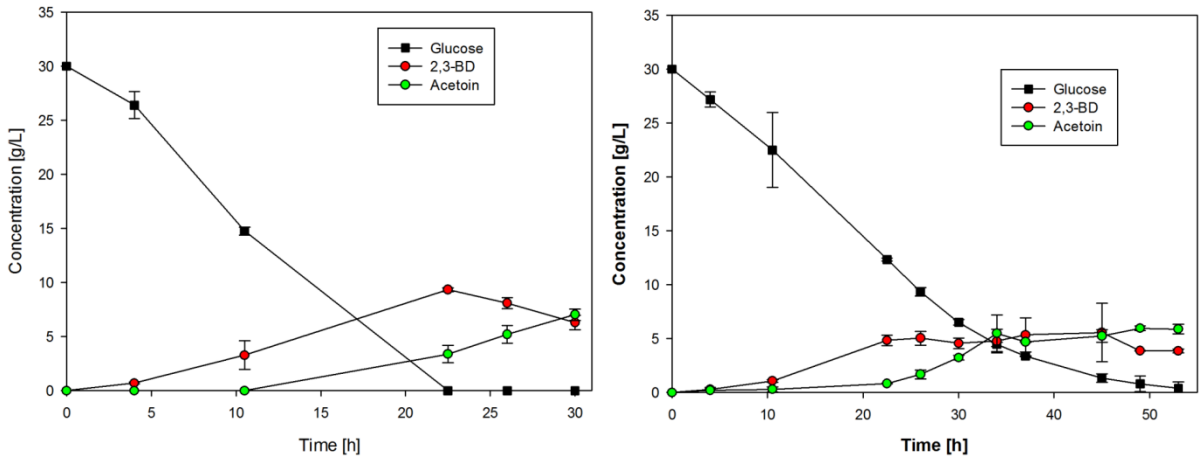


Figure 3-28 Comparison of the cultivation of *B. licheniformis* DSM 8785 using yeast extract / tryptone (5 g/L each; left) and urea (2.73 g/L; right). Conditions: 500 mL shake flasks, 100 mL medium (30 g/L glucose), 30 °C, 100 rpm, initial pH 6.6 (not adjusted) [joint work with Zhou, 2012]

Table 3-16 Comparison of the cultivation of *B. licheniformis* DSM 8785 using yeast extract / tryptone (5 g/L each; left) and urea. Conditions: 500 mL shake flasks, 100 mL medium (100 g/L glucose), 25-35 °C, 100 rpm and initial pH 6.6 (not adjusted) [joint work with Zhou, 2012]

Nitrogen source	Max. 2,3-BD [g/L]	2,3-BD yield [g/g]	Productivity [g/(L*h)]	Acetoin [g/L]
Yeast extract + tryptone	**9.3**	**0.31**	**0.41**	3.4
Urea	5.5	0.27	0.21	0.8

3.3.1.4 Reduction of the amount of yeast extract and tryptone

In chapter 3.3.1.3, the cultivations with yeast extract and tryptone or urea as subtitute were presented. Results obtained during cultivation with urea showed a lower 2,3-BD production compared to using complex medium components. Therefore, a further attempt in reducing the cost for the culture medium was carried out by reducing the amount of yeast extract and tryptone. Three different experimental series were conducted, reducing the amount of both complex medium components simultaneously (Tab. 3-17; A) or reducing the amount of one of the components, while keeping the concentration of the other component constant (Tab. 3-17; B and C).

Table 3-17 Overview of employed yeast extract / tryptone amounts [joint work with Zhou, 2012]

Experimental series	Yeast extract [g/L]	Tryptone [g/L]
A – Reduction of the amount of both components		
	5.0	5.0
	3.75	3.75
	2.5	2.5
	1.25	1.25
	0.0	0.0
B – Reduction of yeast extract; constant tryptone amount		
	5.0	5.0
	3.75	5.0
	2.5	5.0
	1.25	5.0
	0.0	5.0
C – Reduction of tryptone; constant yeast extract amount		
	5.0	5.0
	5.0	3.75
	5.0	2.5
	5.0	1.25
	5.0	0.0

The results of the three experimental series are summarized in Fig. 3-29. With an amount of 5 g/L yeast extract and 5 g/L tryptone, a maximum concentration of 46.1 g/L 2,3-BD was obtained. A reduction of the amount of both yeast extract and tryptone resulted in a corresponding decrease in 2,3-BD production. With an amount of yeast extract and tryptone

reduced to 3/4, 1/2 and 1/4 of the original concentration (3.75 g/L, 2.5 g/L and 1.25 g/L, respectively), 2,3-BD maximum concentration decreased to 33.6 g/L, 27.3 g/L and 12.8 g/L, respectively (Fig. 3-29; A). During the cultivation without addition of complex medium components, only 0.15 g/L 2,3-BD could be detected.

The second experimental series was conducted by keeping the tryptone amount constant at 5 g/L and reducing only the concentration of yeast extract (Fig. 3-29; B). Maximum 2,3-BD production decreased by 5 g/L, when yeast extract was reduced to 3.75 g/L. With 1.25-2.5 g/L yeast extract, 35.5-36.5 g/L 2,3-BD could be detected. The cultivation performed without yeast extract, led to a maximum amount of 27 g/L 2,3-BD.

In the third experimental series, a reduction in the amount of tryptone was carried out, while keeping the amount of yeast extract constant at 5 g/L (Fig. 3-29; C). Maximum 2,3-BD production decreased by 5-6 g/L, each time the amount of yeast extract was reduced to 3.75 g/L, 2.5 g/L and then 1.25 g/L. The cultivation carried out without addition of tryptone yielded 21 g/L 2,3-BD.

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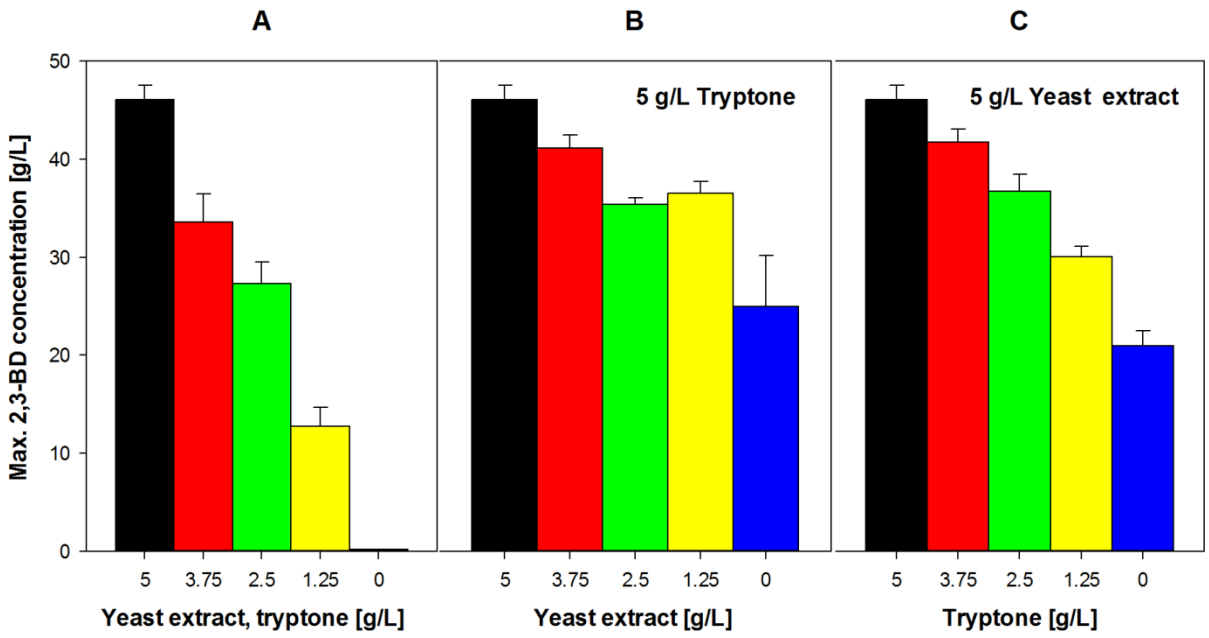

Figure 3-29 Comparison of 2,3-BD production during cultivation of *B. licheniformis* DSM 8785 using different amounts of yeast extract (YE) and tryptone (T): (A) simultaneous reduction of YE and T amounts; (B) reduction of YE amount; 5 g/L T; (C) reduction of T amount; 5 g/L YE. Conditions: 100 mL shake flasks, 20 mL medium (120 g/L glucose), 30 °C, 100 rpm, initial pH 6.6 (not adjusted), 48 h. OD_{540} values: 25.5-0 (A); 25.5-17.5 (B); 25.5-14.2 (C) [joint work with Zhou, 2012]

3.3.1.5 Fed-batch cultivations

The results of batch cultivations with different amounts of glucose using *B. licheniformis* DSM 8785 were presented in chapter 3.3.1.1. With 120 g/L glucose, the highest productivity was identified with a value of 0.99 g/(L*h). During this cultivation, 47 g/L 2,3-BD and a yield of 0.4 g/g glucose were obtained. The glucose concentration of 120 g/L was employed for further experiments using the fed-batch cultivation mode. For the feeding steps, glucose or glucose and all other medium components were added in solid form to the culture broth.

Fig. 3-30 shows the time course for a fed-batch cultivation of *B. licheniformis* DSM 8785. The cultivation was started with a glucose concentration of 120 g/L and incubated at 30°C and 100 rpm. After 40 h, the glucose amount in the culture broth decreased to 10 g/L and 6 g glucose were added in solid form (for a working volume of 100 mL). The working volume reduced by sampling and evaporation was adjusted to 100 mL by addition of sterile Milli-Q water and the culture was incubated under the same conditions as before. A second feeding step was carried out after 64 h, when glucose concentration reached 32 g/L. A maximum amount of 2,3-BD of 46.1 g/L was obtained after 40 h. After the first feeding, 2,3-BD production decreased and acetoin concentration increased to 40 g/L after 64 h. After the second feeding step, acetoin levels dropped and 2,3-BD reached again an amount of 44 g/L after 95 h. After each feeding, there were no significant changes in OD values.

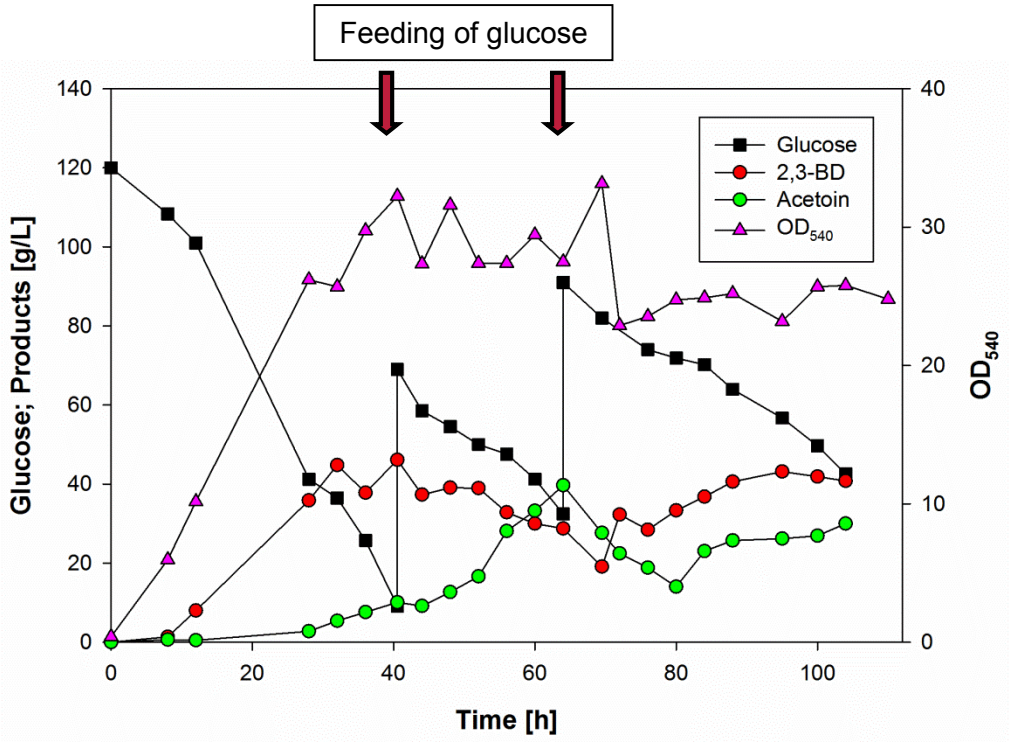

Figure 3-30 Time course for the fed-batch cultivation with *B. licheniformis* DSM 8785. Conditions: 500 mL shake flasks, 100 mL medium (120 g/L glucose), 30°C, 100 rpm, initial pH 6.6 (not adjusted); feeding time points for glucose are indicated by arrows [joint work with Zhou, 2012]

Since 2,3-BD production could not be enhanced by feeding glucose, a second fed-batch cultivation was performed using a combination of glucose and all other medium components (yeast extract, tryptone, salt) for the feeding steps. The time course for this cultivation is illustrated in Fig. 3-31. For each feeding step (38 h, 57 h and 77.5 h), 6 g glucose and corresponding amount of all other nutrients (for a working volume of 100 mL) were added to the culture broth in solid form. With each feeding step, an increase in OD values could be noted. Furthermore, 2,3-BD production increased continuously, reaching a maximum concentration of 115.8 g/L after 97 h. This corresponds to a productivity of 1.19 g/(L*h) and a yield of 0.44 g/g glucose. Regarding by-product formation, 4.6 g/L acetoin were detected after 97 h. The time course of glycerol production showed a maximum of 15.5 g/L after 87.5 h, after which a decrease in glycerol concentration could be observed.

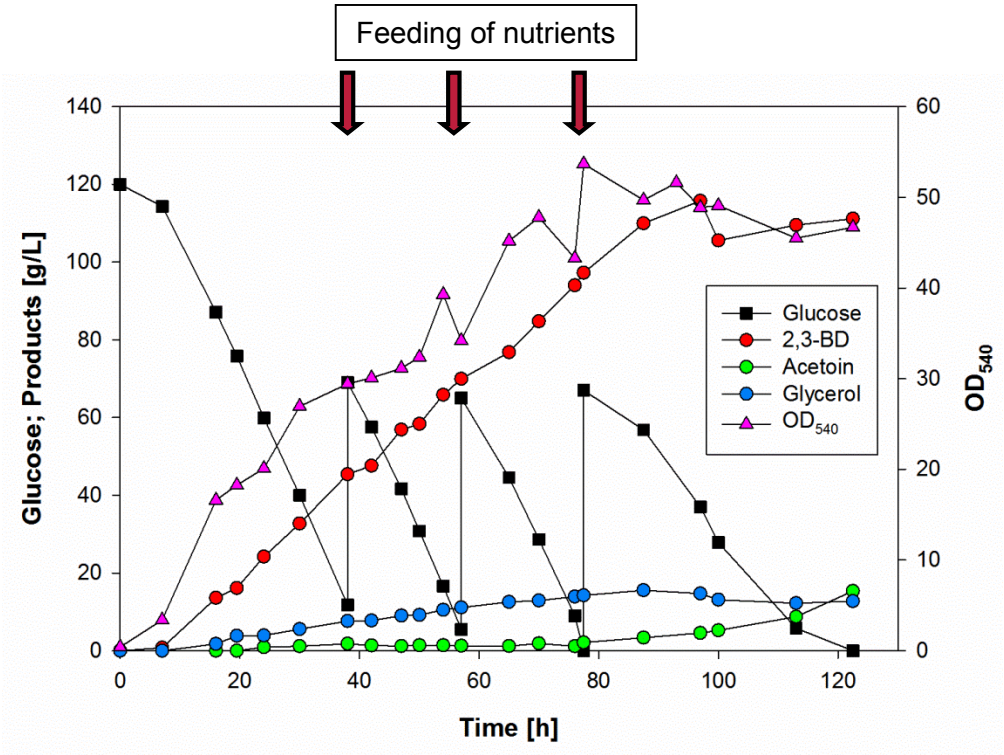

Figure 3-31 Time course for the fed-batch cultivation with *B. licheniformis* DSM 8785. Conditions: 500 mL shake flasks, 100 mL medium (120 g/L glucose), 30°C, 100 rpm, initial pH 6.6 (not adjusted); feeding time points for glucose and other nutrients are indicated by arrows [joint work with Zhou, 2012]

Due to the high 2,3-BD concentration (72.6 g/L) and yield (0.42 g/g glucose) obtained during batch cultivations on medium with 180 g/L glucose, further fed-batch experiments were conducted using this initial glucose concentration. When the glucose concentration decreased to half of the initial concentration, feeding of nutrients was performed. A total amount of 18 g glucose (per 100 mL medium) and corresponding amounts of all other medium components were supplemented in two steps.

The time course of cell growth, glucose consumption, 2,3-BD, acetoin and glycerol production in this fed-batch experiment is shown in Fig. 3-32.

After 44 h the glucose concentration was around 100 g/L and the first feeding step was performed, raising the level of glucose back to the initial concentration. After 69.5 h the amount of glucose decreased to 95 g/L, when the second feeding step was carried out. 2,3-BD production continued increasing after each feeding step and a maximum concentration of 144.7 g/L was obtained after 127 h, when the entire glucose in the culture broth was consumed. This corresponds to a 2,3-BD yield of 0.40 g/g glucose and a productivity of 1.14 g/(L*h). As for by-product formation, acetoin, lactate, acetate and ethanol concentrations below 4 g/L could be detected. The main by-product was glycerol, with a maximum concentration of 18.2 g/L after 127 h.

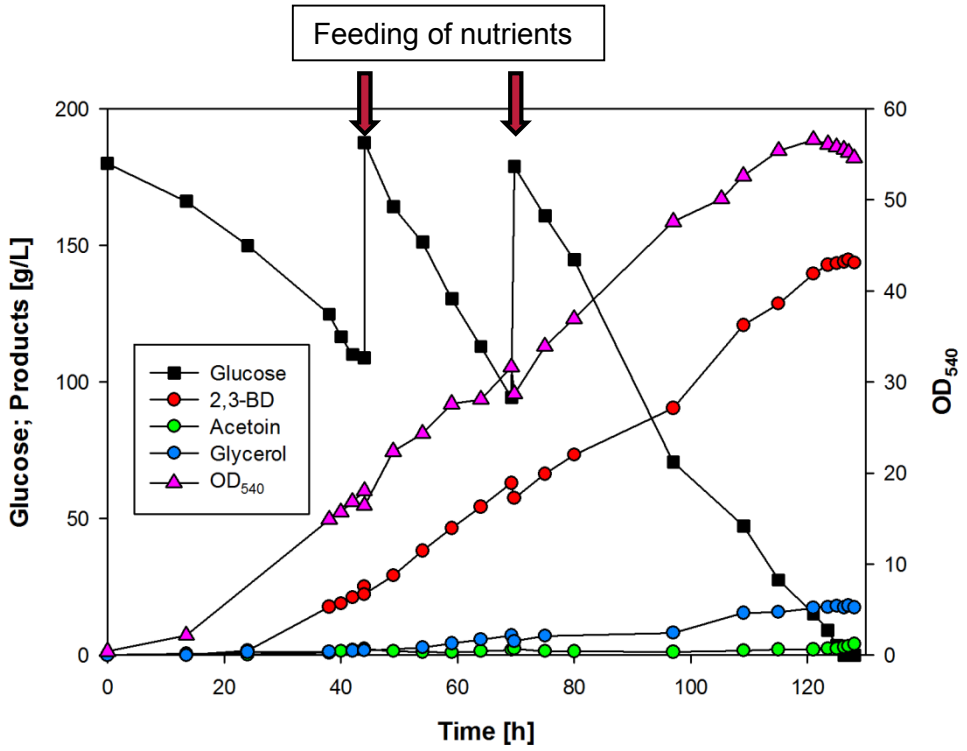

Figure 3-32 Time course for the fed-batch cultivation with *B. licheniformis* DSM 8785. Conditions: 500 mL shake flasks, 100 mL medium (120 g/L glucose), 30°C, 100 rpm, initial pH 6.6 (not adjusted); feeding time points for glucose and other nutrients are indicated by arrows [joint work with Zhou, 2012]

3.3.1.6 Balance on shake flask cultivations on glucose

The main results of shake flask cultivations on glucose with *B. licheniformis* DSM 8785 are summarized in Tab. 3-18. For the batch cultivation mode, the highest 2,3-BD production and yield were obtained with an initial glucose concentration of 180 g/L. On the contrary, the highest productivity was reached during batch cultivation on medium with 120 g/L initial glucose. All batch experiments were conducted at 30°C and 100 rpm.

Using the fed-batch cultivation mode with feeding of only glucose (initial 120 g/L and 2x feeding of 60 g/L), 2,3-BD production could not be enhanced compared to the corresponding batch experiment. However, an increase in productivity from 0.99 to 1.14 g/(L*h) could be observed. When all other nutrients (yeast extract, tryptone, salt) were used for feeding in addition to glucose, 2,3-BD concentration was increased up to 115.8 g/L (from 120 g/L initial glucose and 3x feeding of 60 g/L glucose and nutrients). An additional increase in 2,3-BD yield to 0.44 g/g glucose and productivity to 1.19 g/(L*h) could be detected. The highest amount of 2,3-BD, 144.7 g/L, was reached during the fed-batch cultivation with an initial glucose concentration of 180 g/L and feeding of an additional amount of 180 g/L glucose and nutrients in two steps.

Table 3-18 Comparison between cultivations with *B. licheniformis* DSM 87851 using the batch / fed-batch cultivation modes [joint work with Zhou, 2012]

Cultivation type	Time [h]	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]
Batch No. 1 *	47.3	47.1	0.40	**0.99**
Batch No. 2 **	86.0	**72.6**	**0.42**	0.86
Fed-batch ** (2x60 g/L glucose)	40.5	46.1	0.42	1.14
Fed-batch ** (3x60 g/L glucose + other nutrients)	97.0	115.8	**0.44**	**1.19**
Fed-batch ** (2x90 g/L glucose + other nutrients)	127.0	**144.7**	0.40	1.14

Cultivation conditions: * 120 g/L glucose; 5 g/L yeast extract; 5 g/L tryptone; 30°C; 100 rpm
** 180 g/L glucose; 5 g/L yeast extract; 5 g/L tryptone; 30°C; 100 rpm

3.3.2 Shake flask experiments on wood hydrolysates

In addition to experiments on pure sugars, further cultivations in the shake flask scale were conducted on wood hydrolysates. Cultivations were performed on natural wood hydrolysate medium, as well as on a specially constructed artificial wood hydrolysate medium. The artificial medium was also employed for investigating the effect of potential inhibitory compounds present in natural wood hydrolysates on bacterial growth and 2,3-BD production.

3.3.2.1 Comparison between cultivations on natural and artificial wood hydrolysates

Parallel cultivations were carried out on natural and artificial wood hydrolysate medium. The natural wood hydrolysate was obtained from the fiber fraction of poplar wood and contained 30 g/L glucose. The composition was given in Tab. 3.8 (chapter 3.2.2.1). The artificial wood hydrolysate medium was constructed based on the identified components of the natural wood hydrolysate (sugars and inhibitors). The results of these experiments are shown in Fig. 3-33 and Tab. 3-19. In the beginning of the cultivation on natural wood hydrolysate medium, glucose consumption and 2,3-D production were slower. After 22 h, a maximum amount of 9.3 g/L 2,3-BD were obtained from artificial medium and 10.5 g/L 2,3-BD using natural wood hydrolysate medium. Yield and productivity are higher from natural wood hydrolysates, but both media show a similar behavior regarding 2,3-BD production, in spite of the small concentration difference.

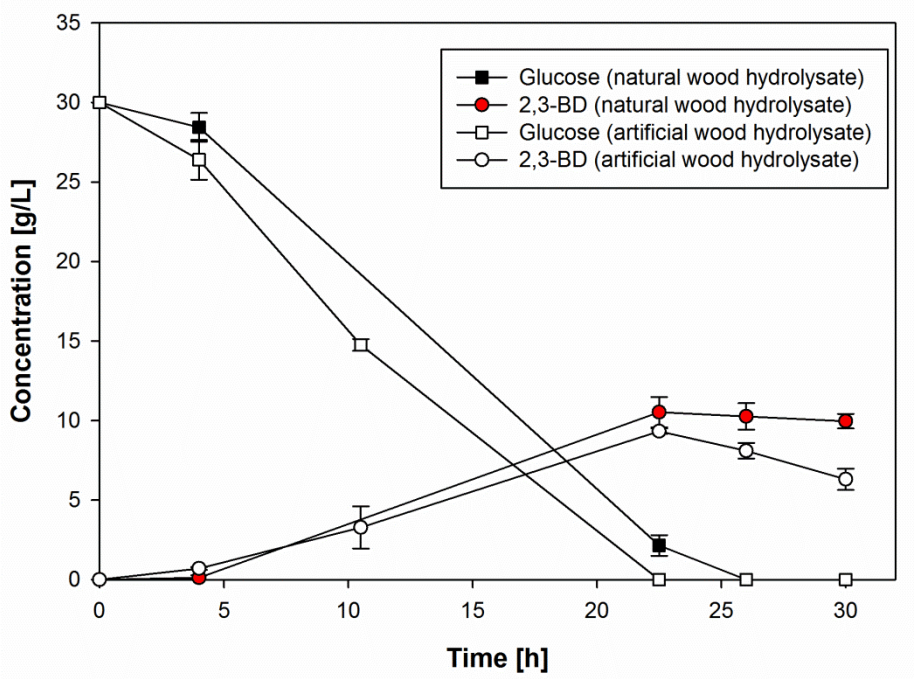

Figure 3-33 Comparison of *B. licheniformis* DSM 8785 cultivation on natural and artificial wood hydrolysate medium. Conditions: 500 mL shake flasks, 100 mL medium (30 g/L glucose), 30 °C, 100 rpm, initial pH 6.6 (not adjusted) [joint work with Zhou, 2012]

Table 3-19 Comparison between cultivations using *B. licheniformis* DSM 8785 on natural and artificial wood hydrolysate medium [joint work with Zhou, 2012]

Medium	Time [h]	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]
Natural wood hydrolysate	22	10.5	0.38	0.47
Artificial wood hydrolysate	22	9.3	0.31	0.41

3.3.2.2 Effect of potential inhibitory compounds from natural wood hydrolysates

As mentioned in chapter 3.2.2.2, several potential inhibitory compounds are present in natural wood hydrolysates. These compounds may potentially inhibit bacterial growth, sugar consumption and 2,3-BD production. The influence of these compounds was investigated using artificial wood hydrolysate medium with 120 g/L glucose. The potential inhibitory compounds were tested separately or combined in various concentrations (e.g. 0.5-fold, 1-fold, 2-fold, 4-fold, 8-fold, 16-fold). The 1-fold concentration corresponds to the concentration of these compounds in natural wood hydrolysate medium with 120 g/L glucose. The tested potential inhibitory compounds and the corresponding amounts are given in Tab. 3-20.

Table 3-20 Concentrations of potential inhibitory compounds corresponding to natural wood hydrolysates with 120 g/L glucose and 2-fold-16-fold amounts [joint work with Zhou, 2012]

	Concentrations [g/L]					
Compound	**0.5-fold**	**1-fold**	**2-fold**	**4-fold**	**8-fold**	**16-fold**
Furfural	0.06	0.12	0.24	0.48	0.96	1.92.
5-Hydroxymethylfurfural	0.08	0.16	0.32	0.64	1.28	2.56
4-Hydroxybenzoic acid	0.15	0.30	0.60	1.20	2.40	4.80
Vanillin	0.007	0.014	0.028	0.056	0.112	0.224
Syringaaldehyde	0.01	0.02	0.04	0.08	0.16	0.32
Formic acid	0.60	1.20	2.40	4.80	9.60	19.20
Acetic acid	1.80	3.60	7.20	14.40	28.80	57.60

As an example, the time course for glucose consumption and 2,3-BD production during cultivation on medium containing 120 g/L glucose and 0-19.2 g/L formic acid is illustrated in Fig. 3-34. During the cultivation without addition of formic acid, the entire glucose was consumed after 48 h of cultivation. In the presence of 0.6 g/L formic acid (0.5x concentration),

glucose consumption was slower, but also complete after 48 h. With increasing levels of formic acid, the decrease of glucose amount in the culture medium was progressively slower. The cultivation performed with the highest concentration of the inhibitor (19.2 g/L) showed almost no reduction of glucose concentration over the entire cultivation time and 2,3-BD production remained below 1 g/L . In the absence of the inhibitor, 46.5 g/L 2,3-BD were produced after 43 h, while in the presence of 0.6 g/L formic acid 46.3 g/L 2,3-BD could be detected. Production was slower when 1.2 g/L formic acid were added to the culture medium, reaching maximum levels of 42.9 g/L 2,3 BD after 48 h. With concentrations of 2.4-9.6 g/L formic acid, a significant reduction in product formation was observed, with maximum amounts of 19.3-21.1 g/L after 48 h.

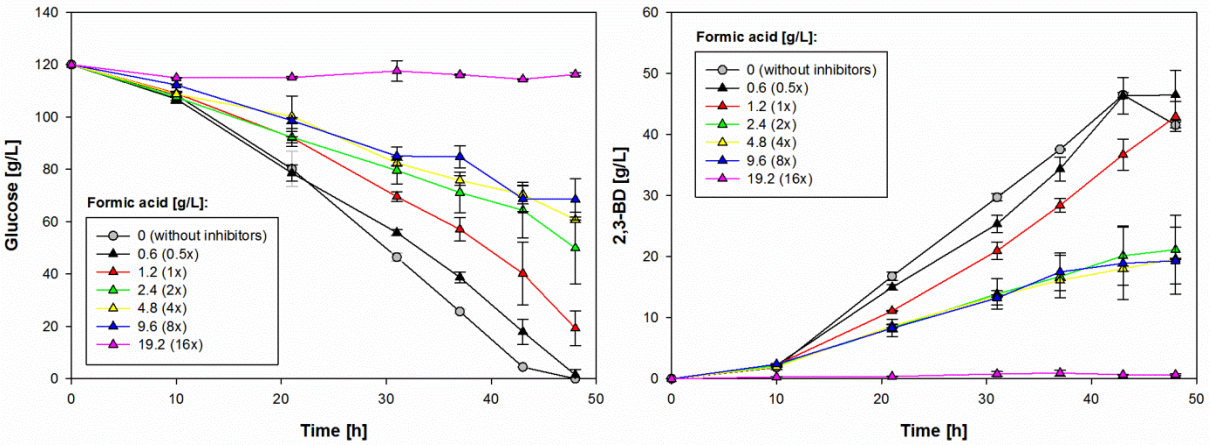

Figure 3-34 Glucose consumption (left) and 2,3-BD production (right) during cultivation of *B. licheniformis* DSM 8785 on medium with 0-19.2 g/L formic acid. Conditions: 100 mL shake flasks, 20 mL medium (120 g/L glucose), 30°C, 100 rpm [joint work with Zhou, 2012]

The studies performed with addition of combined inhibitory compounds showed different results, depending on the amount of the added substances (Fig. 3-35). The entire glucose was consumed after 48 h in the presence of 0.5x amount of inhibitors as well as during the cultivation without inhibitors. After 43 h, maximum 2,3-BD production reached 46.5 g/L (without inhibitors) and 44.5 g/L (0.5x concentration of compounds). With an increased amount of compounds (1x-2x concentrations), an inhibiting effect could be observed. After 48 h, an amount of 50-55 g/L residual glucose was still present in the culture broth. Maximum 2,3-BD concentrations obtained were between 14-18 g/L. A further increase in the concentration of inhibitory compounds to 2.5x-3x led to a complete inhibition of glucose consumption and subsequently no 2,3-BD could be produced.

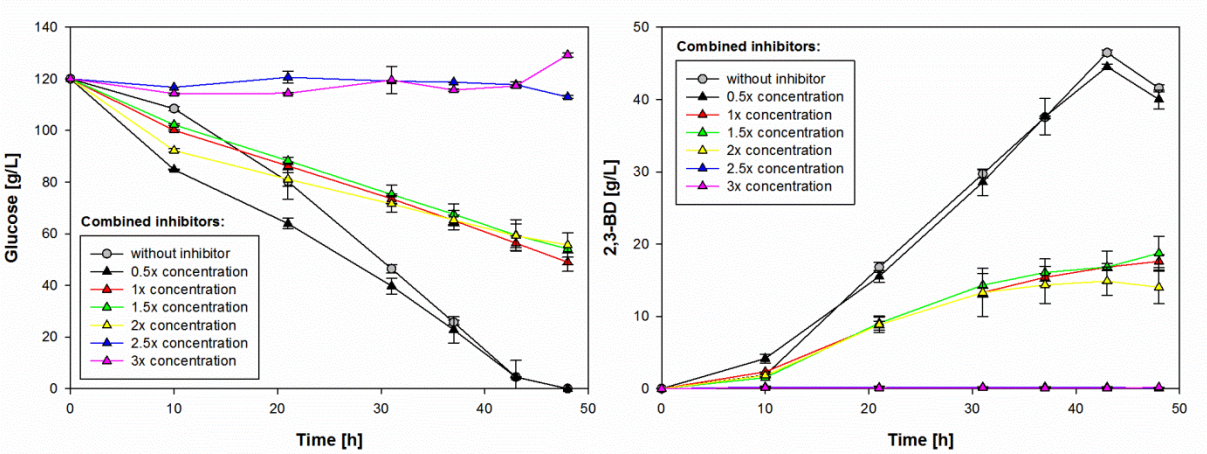

Figure 3-35 Glucose consumption (left) and 2,3-BD production (right) during cultivation of *B. licheniformis* DSM 8785. Conditions: 100 mL shake flasks, 20 mL medium (120 g/L glucose and combined inhibitory compounds in 0.5x-3x concentrations), 30°C, 100 rpm [joint work with Zhou, 2012]

3.3.2.3 Balance on the effect of potential inhibitory compounds

Tab. 3-21 summarizes the results of shake flask cultivations on artificial wood hydrolysate medium with addition of various amounts of potential inhibitory compounds. No inhibiting effect could be detected during cultivation with 1/2 the amount of compounds present in natural wood hydrolysates with 120 g/L glucose. With higher concentrations of compounds, an inhibiting effect on glucose consumption and 2,3-BD production could be observed.

Table 3-21 Balance on the effect of potential inhibitory compounds in natural wood hydrolysates. No inhibition (-), inhibition (+), strong inhibition (++) [joint work with Zhou, 2012]

	Inhibition at following concentrations:					
Compound	**0.5-fold**	**1-fold**	**2-fold**	**4-fold**	**8-fold**	**16-fold**
Furfural	-	-	-	-	+	++
5-Hydroxymethylfurfural*	-	-	-	-	-	-
4-Hydroxybenzoic acid**	-	-	-	-	++	++
Vanillin***	-	-	-	-	-	-
Syringaaldehyde****	-	-	-	-	-	-
Formic acid	-	-	+	+	+	++
Acetic acid*****	-	-	-	-	++	++
Combined inhibitors	-	+	+	++	++	++

* inhibition from 32x amount (5.12 g/L); strong inhibition from 64x amount (10.24 g/L)
** inhibition from 6x amount (1.8 g/L)
*** inhibition from 160x amount (2.24 g/L); strong inhibition from 192x amount (2.69 g/L)
**** inhibition from 40x amount (0.8 g/L); strong inhibition from 64x amount (1.28 g/L)
***** inhibition from 4.25x amount (15.3 g/L)

3.3.3 Scale-up to the 3.5 L bioreactor scale on different C-sources

In this chapter the results of cultivations in the 3.5 L bioreactor scale are presented. The optimized parameters from the shake flask scale were employed and further optimization steps were carried out. The experiments were conducted on medium with different C-sources: glucose, artificial wood hydrolysate and natural wood hydrolysate. On artificial wood hydrolysate medium, cultivations were performed both with and without addition of potential inhibitory compounds. The aim of the bioreactor experiments was to optimize the cultivation parameters in order to reproduce the results obtained in the shake flask scale.

3.3.3.1 Influence of the impeller speed on 2,3-BD production

The first bioreactor cultivation using *B. licheniformis* DSM 8785 was conducted on medium with 100 g/L glucose at 30°C, 200 rpm and 0.5 L/(L*min) aeration rate (based on Nakashimada et al. 2000). Fig. 3-36 shows the time course for the corresponding bioreactor cultivation. Bacterial growth, glucose consumption and 2,3-BD production were slow during the first 24 h of cultivation. By increasing the stirring speed to 400 rpm (after 24.25 h) and then 500 rpm (after 34 h), bacterial growth was increased, reaching a maximum OD_{540} value of 25 after 50.75 h. Furthermore, glucose consumption and 2,3-BD production increased. Maximum product concentration (26 g/L) was obtained after 50.75 h, while 19.5 g/L residual glucose was measured. Acetoin production showed a significant increase from 2 to 9.9 g/L, when the stirring speed was increased from 400 to 500 rpm.

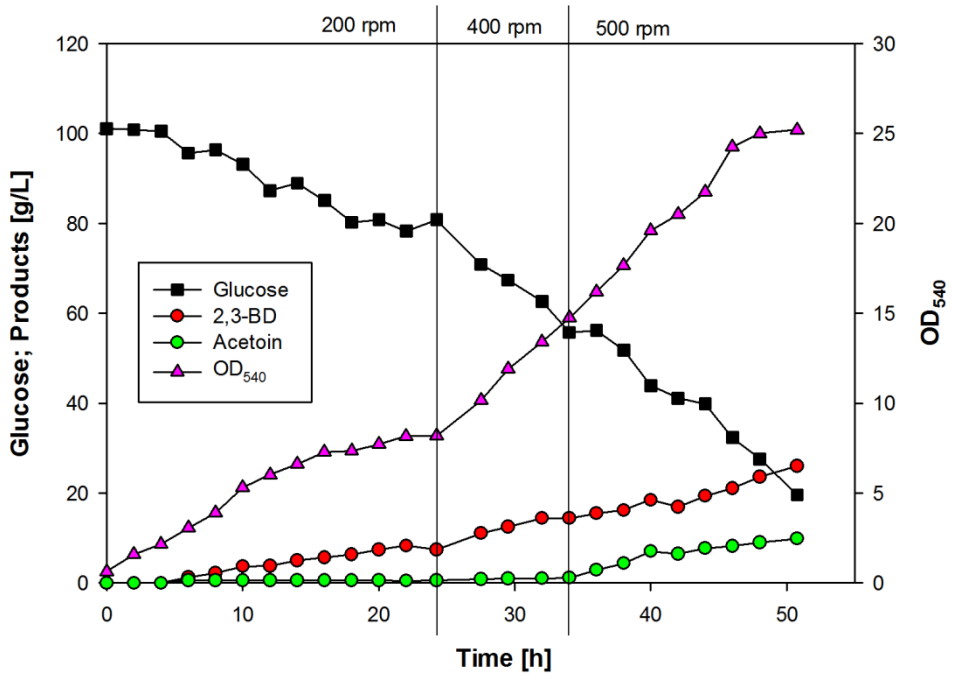

Figure 3-36 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 3.5 L Minifors bioreactor, 2.8 L medium (100 g/L glucose), 200 mL preculture, 30°C, 200-500 rpm, aeration rate 0.5 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Hamann, 2010]

3.3.3.2 Effect of different aeration rates on 2,3-BD production

With a stirring speed of 400 rpm further bioreactor experiments were conducted, in order to detect the optimum aeration rate. An artificial wood hydrolysate medium (without inhibitors) containing 120 g/L glucose, 11 g/L xylose and 1.1 g/L mannose was employed for cultivation. Three different aeration rates were tested: 0.8 L/(L*min), 1.2 L/(L*min) and 1.5 L/(L*min).

Fig. 3-37 illustrates the time course for the bioreactor cultivation of *B. licheniformis* DSM 8785 on artificial wood hydrolysate medium with 120 g/L glucose at 30°C, 400 rpm and with an aeration rate of 1.2 L/(L*min). After a 4 h lag phase, bacterial growth started and maximum OD_{540} values of 30 were obtained after 45 h. Glucose consumption was slow during the first 14 h, after which the consumption increased significantly and remained constant throughout the rest of the cultivation. The entire amount of glucose was completely consumed after 56 h. 2,3-BD production started after 10 h and proceeded with a constant rate, reaching a maximum concentration of 47.6 g/l after 56 h. Culture pH fluctuated between 5.9 and 6.6. Acetoin levels remained below 2 g/L during the first 34 h; at the time point of maximum 2,3-BD concentration, 7.1 g/L acetoin were measured in the culture broth. At the same time, the highest amount of glycerol was 4.3 g/L. Additionally, 1.2 g/L ethanol were obtained (results not shown).

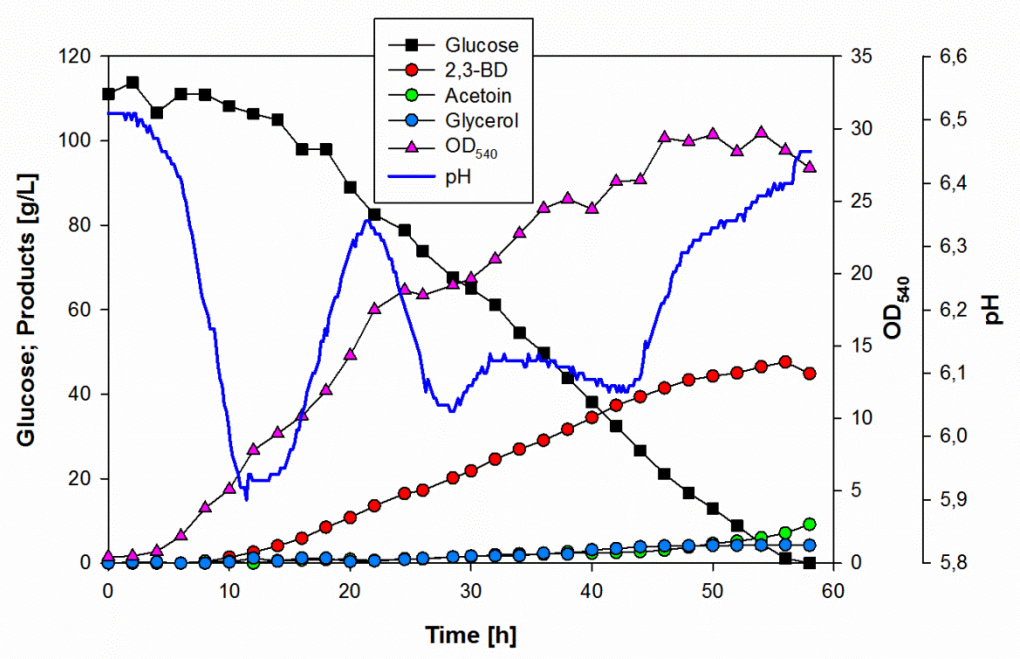

Figure 3-37 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (120 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Zhou, 2012]

Tab. 3-22 summarizes the results of the bioreactor experiments with *B. licheniformis* DSM 8785 on artificial wood hydrolysate medium (without inhibitors) at different aeration rates. By increasing the aeration rate from 0.8 to 1.2 L/(L*min), an increase in maximum 2,3-BD production to 47.6 g/L, 2,3-BD yield to 0.43 g/g glucose as well as an enhanced productivity of 0.85 g/(L*h) could be observed. With an aeration rate of 1.5 L/(L*min), 2,3-BD concentration, yield and productivity were in the same range as with an aeration rate of 0.8 L/(L*min). Optimum conditions for batch cultivation were 30°C, 400 rpm and 1.2 L/(L*min) aeration rate.

Table 3-22 Comparison between bioreactor cultivations of *B. licheniformis* DSM 8785 on artificial wood hydrolysate medium (120 g/L glucose; without inhibitors) using different aeration rates [joint work with Zhou, 2012]

Aeration rate [L/(L*min)]	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]	Acetoin [g/L]	Glycerol [g/L]
0.8	43.6	0.37	0.81	5.4	6.5
1.2	47.6	0.43	0.85	7.1	4.3
1.5	42.2	0.35	0.75	13.5	1.0

3.3.3.3 Cultivations on artificial wood hydrolysates with / without inhibitors

As already investigated in the shake flask scale, cultivation experiments on artificial wood hydrolysates with and without addition of inhibitory compounds were carried out. Regarding the aeration rate, the value of 1.2 L/(L*min) was employed. The results of the cultivation on artificial wood hydrolysate medium without inhibitors were shown in chapter 3.3.3.2 and summarized in Tab. 3-22.

The time course for bacterial growth, glucose consumption, product formation and pH for the cultivation on artificial wood hydrolysates with addition of inhibitors is shown in Fig. 3-38. The amount of inhibitors added to the medium corresponds to the 1-fold concentration of compounds described in Tab. 3-20. After a short lag phase, bacterial growth started and a maximum OD_{540} value of 24.2 was obtained after 46 h. The complete amount of glucose present in the culture medium was consumed after 58 h, when a maximum 2,3-BD concentration of 46.1 g/L was reached. Acetoin production increased slowly during cultivation; at the time point of maximum 2,3-BD production the corresponding amount of acetoin was 6 g/L. Glycerol levels remained below 1 g/L until complete glucose consumption, then rapidly increased to values above 9 g/L. Furthermore, 1.2 g/L ethanol was obtained (results not shown). The pH value fluctuated between 6.6 and 8.4, showing an increasing tendency during the entire cultivation.

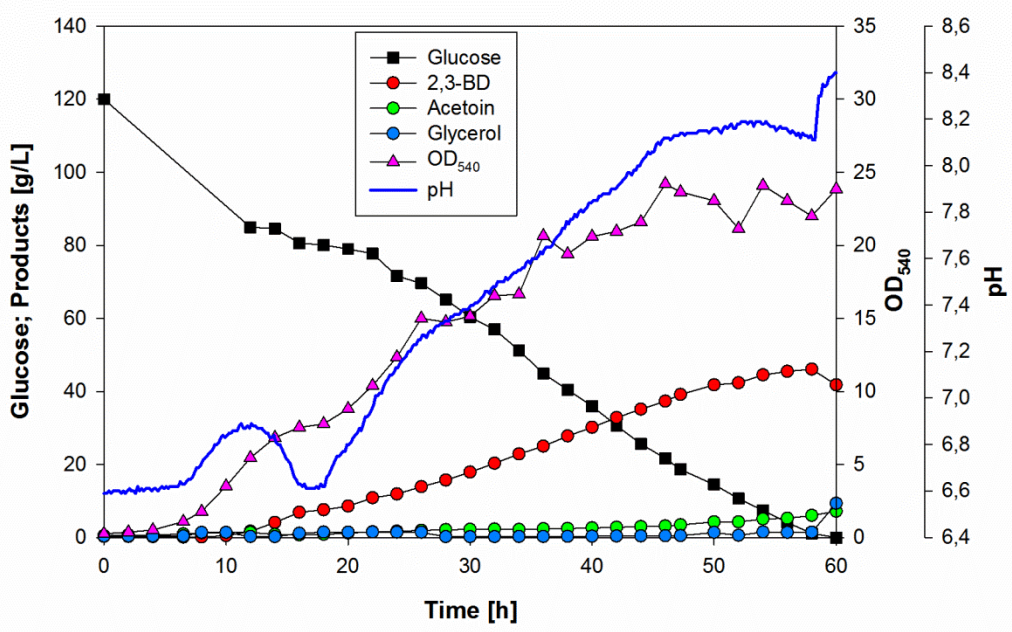

Figure 3-38 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (120 g/L glucose; 1-fold concentration of inhibitors), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Zhou, 2012]

A comparison of the results obtained during cultivation with *B. licheniformis* DSM 8785 in the 3.5 L bioreactor scale on artificial wood hydrolysate (with 120 g/L glucose) with / without addition of potential inhibitory compounds is given in Tab. 3-23. Concerning maximum 2,3-BD production no significant difference could be observed between the two cultivations. Yield and productivity were also in the same range, but somewhat lower in the presence of inhibitory compounds.

Table 3-23 Comparison between bioreactor cultivations of *B. licheniformis* DSM 8785 on artificial wood hydrolysate medium with / without addition of inhibitors [joint work with Zhou, 2012]

Artificial wood hydrolysate medium	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]	Acetoin [g/L]	Glycerol [g/L]
Without inhibitors	47.6	0.43	0.85	7.1	4.3
With inhibitors	46.1	0.39	0.79	6.0	1.4

3.3.3.4 Cultivation on natural wood hydrolysate

Apart from the cultivations performed on medium containing pure glucose or artificial wood hydrolysate, natural wood hydrolysates were employed as sugar source for 2,3-BD production in the 3.5 L bioreactor scale. A natural wood hydrolysate obtained from the fiber fraction of poplar wood (containing 216.75 g/L glucose) was delivered by our project partner from the Institute of Wood Technology of the Thünen-Institute. The composition of the natural wood hydrolysate is given in Tab. 3-24 (left). The other nutrients were added and the hydrolysate was diluted to an initial glucose concentration of 120 g/L, in order to create similar conditions to the cultivation on artificial wood hydrolysate. In contrast to the cultivations using pure sugars, a second preculture on natural wood hydrolysate was necessary. The concentration of sugars and inhibitory compounds after further dilution of the medium by addition of 200 mL preculture is shown in Tab. 3-24 (right).

Table 3-24 Identified components of natural wood hydrolysate (fibre fraction of poplar wood) delivered by the Institute of Wood Technology of the Thünen-Institute (Hamburg-Harburg)

Compounds	Concentrations [g/L]	
	In original hydrolysate	After dilution
Glucose	216.75	100.6
Xylose	7.71	3.58
Mannose	1.65	0.77
Furfural	0.0	0.0
5-Hydroxymethylfurfural	0.310	0.144
4-Hydroxybenzoic acid	0.568	0.264
Vanillin	0.019	0.009
Syringaaldehyde	0.038	0.018
Formic acid	0.185	0.086
Acetic acid	1.667	0.774

Fig. 3-39 shows the time course for the cultivation on natural wood hydrolysate with a glucose concentration of 100.6 g/L. During the first 15 hours, glucose consumption was slow; afterwards the consumption increased and remained constant until no residual glucose was left in the medium (after 70 h). Bacterial growth started after 8 h and the highest OD_{540} was reached after 60-70 h with a value of 26.2. Maximum 2,3-BD levels of 36 g/L were measured after 70 h. Regarding by-product formation, 1.5 g/L acetoin, 5.3 g/L glycerol and 6.8 g/L ethanol could be detected at the time point of maximum 2,3-BD production. The pH curve showed an increasing tendency during the entire cultivation from initially 6.6 to 7.6.

A summary of the results obtained during cultivation with *B. licheniformis* DSM 8785 in the 3.5 L bioreactor scale on natural and artificial wood hydrolysate (with addition of inhibitors) is given in Tab. 3-25. Maximum 2,3-BD production was much lower during cultivation on natural wood hydrolysate. However, the difference in the initial glucose concentration should not be disregarded. Glucose consumption was 10 h slower on natural wood hydrolysate medium, leading to a lower productivity. Furthermore, the concentrations of by-products (e.g. ethanol, glycerol) were higher on natural wood hydrolysate medium; this corresponded to a lower 2,3-BD yield.

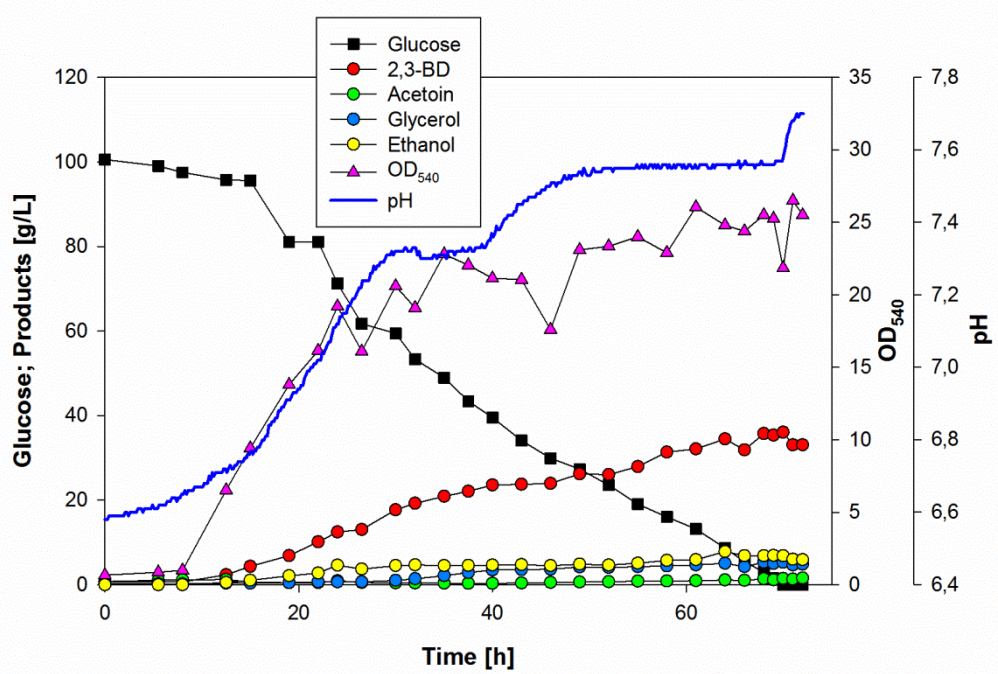

Figure 3-39 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 3.5 L Minifors bioreactor, 2.8 L natural wood hydrolysate medium (100.6 g/L glucose; concentration of inhibitors according to Tab. 3-24), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Zhou, 2012]

Table 3-25 Comparison between bioreactor cultivations of *B. licheniformis* DSM 8785 on artificial and natural wood hydrolysate medium [joint work with Zhou, 2012]

Wood hydrolysate medium	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]	Acetoin [g/L]	Glycerol [g/L]	Ethanol [g/L]
Artificial 120 g/L glucose	47.6	0.43	0.85	7.1	4.3	1.2
Natural 100.6 g/L glucose	36.0	0.36	0.51	1.5	5.3	6.8

3.3.3.5 Fed-batch cultivations on artificial wood hydrolysate without inhibitors

During batch cultivations with an initial glucose concentration of 120 g/L glucose, 47.6 g/L 2,3-BD, a yield of 0.43 g/g glucose and a productivity of 0.85 g/(L*h) were obtained. In order to enhance 2,3-BD production, further experiments were conducted using the fed-batch cultivation mode. The initial glucose concentration was 120 g/L and different amounts of glucose and other medium components were added in solid form to the culture broth. Three feeding strategies were tested:

(A) 5 times feeding of 90 g glucose (per 3 L culture) and corresponding amounts of other nutrients at 90 g/L residual glucose;
(B) 5 times feeding of 135 g glucose (per 3 L culture) and corresponding amounts of other nutrients at 75 g/L residual glucose;
(C) 5 times feeding of 180 g glucose (per 3 L culture) and corresponding amounts of other nutrients at 60 g/L residual glucose.

Fig. 3-40 shows the time course for the fed-batch cultivation performed using strategy A. The initial glucose concentration of 120 g/L decreased after 23 h to 93 g/L, when the first feeding step was performed, raising the level of glucose back to the initial concentration. Further feeding steps were carried out after 31 h, 46 h, 55.5 h and 67 h. During each feeding step, the glucose concentration was increased by 30 g/L through addition of 90 g glucose and corresponding amounts of all other nutrients in solid form to the 3 L culture broth.

After a short lag phase, bacterial growth was fast and maximum OD_{540} values around 30 were reached after 85-113 h. The pH decreased from an initial value of 6.6 to 5.9 during the first 20 h and then pH fluctuations were observed. With each feeding step, the pH increased again and then decreased as glucose was consumed.

2,3-BD production started after 20 h and continued increasing after each feeding step with a constant production rate. A maximum concentration of 103.3 g/L 2,3-BD was obtained after 118.25 h, when the entire glucose in the culture broth was consumed. This corresponds to a 2,3-BD yield of 0.37 g/g glucose and a productivity of 0.91 g/(L*h).

Regarding by-product formation, 3.5 g/L acetoin could be detected at the time point of maximum 2,3-BD concentration, while ethanol levels remained below 1 g/L (results not shown). The main by-product was glycerol, with a maximum concentration of 37.7 g/L after 118.25 h. Glycerol production increased constantly after each feeding step, showing a constant production rate during the entire cultivation.

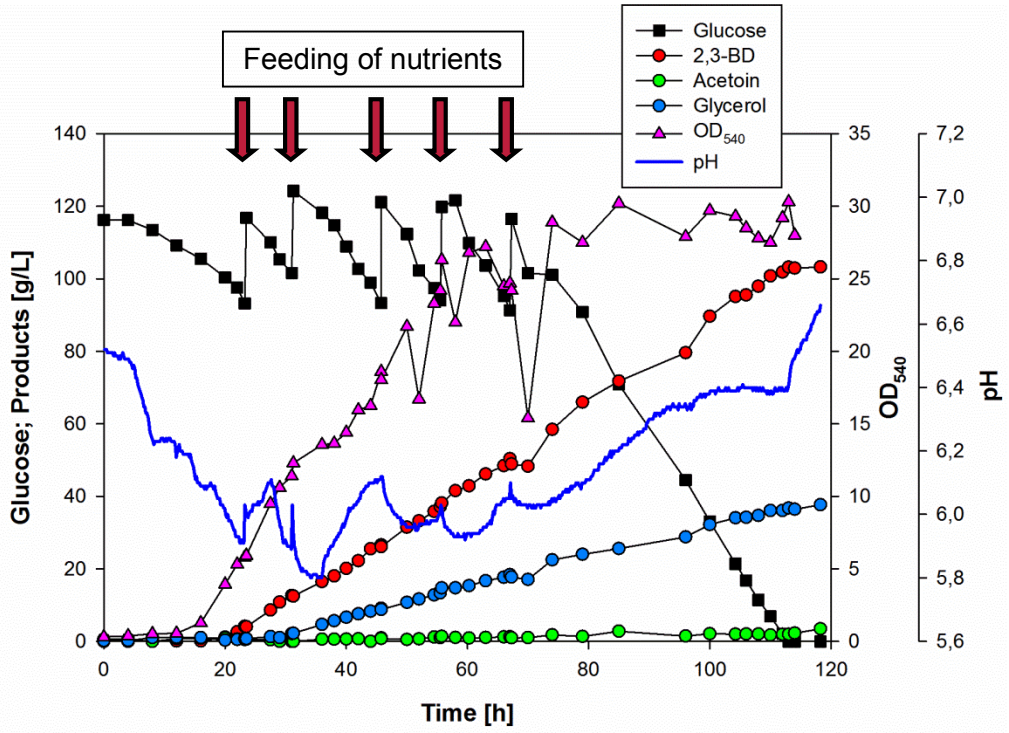

Figure 3-40 Time course for the fed-batch cultivation of *B. licheniformis* DSM 8785 (strategy A). Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (120 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), initial pH 6.6 (not adjusted); feeding time points for glucose (90 g for 3 L working volume) and corresponding amounts of nutrients are indicated by arrows [joint work with Zhou, 2012]

The time course for the fed-batch cultivation performed using feeding strategy B is illustrated in Fig. 3-41 (a). After 23 h, the glucose concentration decreased to 73 g/L and the first feeding step was performed. By addition of 135 g glucose and corresponding amounts of the other nutrients (per 3 L culture), the glucose concentration was increased by 45 g/L with each feeding step. Additional feeding steps were carried out after 33 h, 47.5 h, 60 h and 70 h. The entire amount of glucose was consumed after 121 h. Maximum OD_{540} values around 49 were measured after 74-106 h. The production rate for 2,3-BD was constant after the first 10 h of cultivation. Maximum 2,3-BD levels of 127.4 g/L could be detected after 121 h, corresponding to a yield of 0.37 g/g glucose and a productivity of 1.05 g/(L*h). Glycerol production was high during fed-batch cultivation, yielding maximum amounts of 30 g/L after 121 h. Furthermore, 2.3 g/L acetoin and 0.8 g/L ethanol were measured.

Fig. 3-41 (b) shows the exhaust gas analysis for this fed-batch cultivation. After the lag phase, the pO_2 decreased considerably and the value fluctuated around 20%. OUR and CER increased after each feeding step, indicating an enhanced bacterial growth, glucose consumption and CO_2 production. Corresponding fluctuations were observed for the RQ curve with values between 5 and 7 after the first two feedings and then decreased to 4-5.

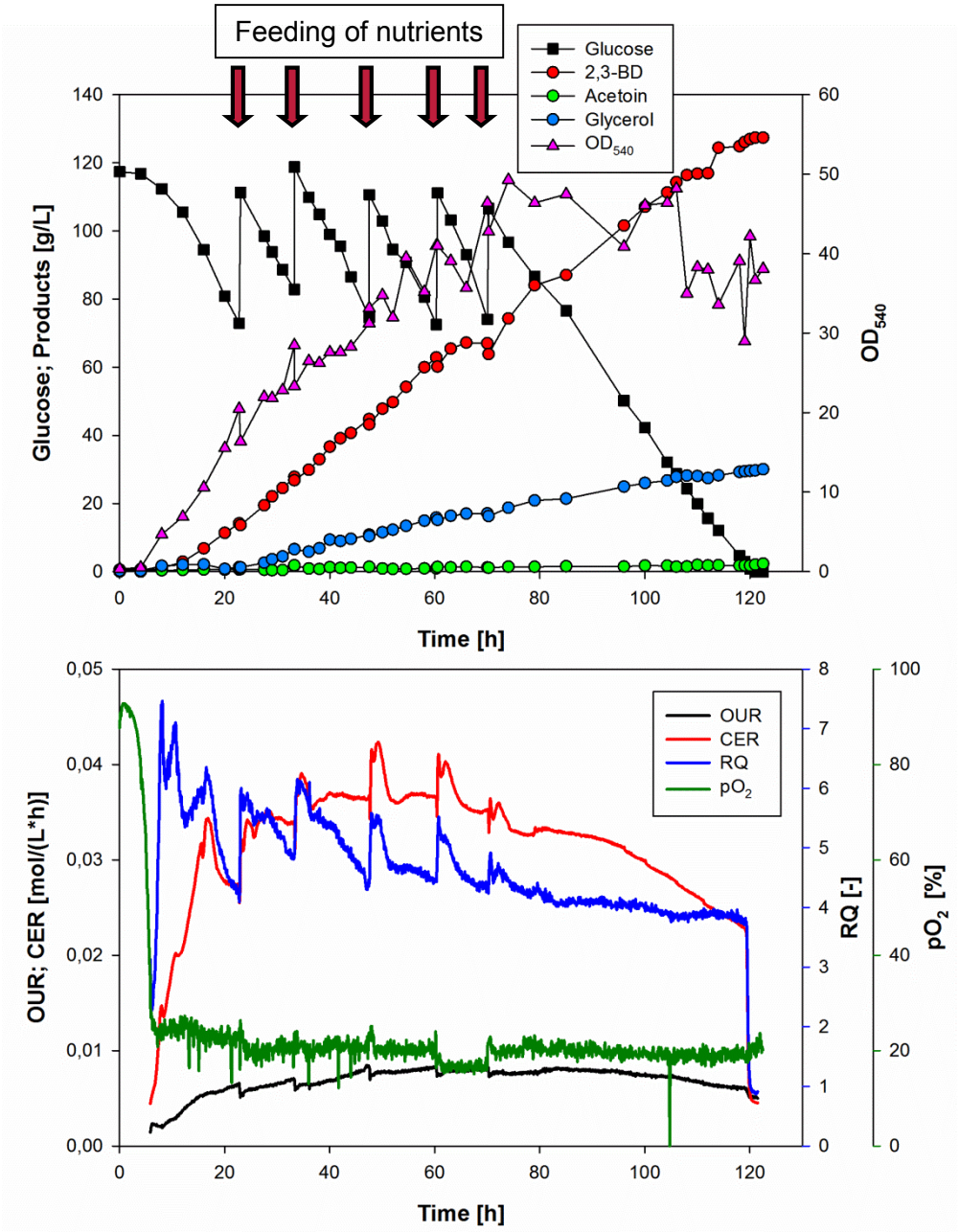

Figure 3-41 Time course for the fed-batch cultivation of *B. licheniformis* DSM 8785 (strategy B).
(a) Bacterial growth, glucose consumption, 2,3-BD and by-product formation;
(b) Exhaust gas analysis.
Conditions: 3.5 L Minifors bioreactor, 2.8 L artificial wood hydrolysate medium (120 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), initial pH 6.6 (not adjusted); feeding time points for glucose (135 g for 3 L working volume) and corresponding amounts of nutrients are indicated by arrows [joint work with Zhou, 2012]

A comparison between the three fed-batch strategies is listed in Tab. 3-26. The highest 2,3-BD production, yield and productivity were reached using strategy B (5 times feeding of 45 g/L glucose and other nutrients at 75 g/L residual glucose). With all strategies, glycerol amounts above 25 g/L could be detected.

Table 3-26 Comparison between fed-batch cultivations with *B. licheniformis* DSM 87851 using three different feeding strategies [joint work with Zhou, 2012]

Strategy	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]	Acetoin [g/L]	Glycerol [g/L]
A (5x30 g/L glucose+ other nutrients)	103.3	0.37	0.91	1.9	36.8
B (5x45 g/L glucose + other nutrients)	127.4	0.37	1.05	2.0	29.7
C (5x60 g/L glucose + other nutrients)	100.6	0.29	0.75	5.7	27.3

3.3.3.6 Cultivations with higher initial glucose concentrations (180 g/L)

Due to the high 2,3-BD concentration (72.6 g/L) and yield (0.42 g/g glucose) obtained in the shake flask scale during batch cultivations on medium with 180 g/L glucose, further experiments were conducted in the 3.5 L bioreactor scale using this initial glucose concentration. Fig. 3-42 shows the time course for batch cultivation using *B. licheniformis* DSM 8785 on medium with 180 g/L glucose at 30°C, 400 rpm and with an aeration rate of 1.2 L/(L*min).

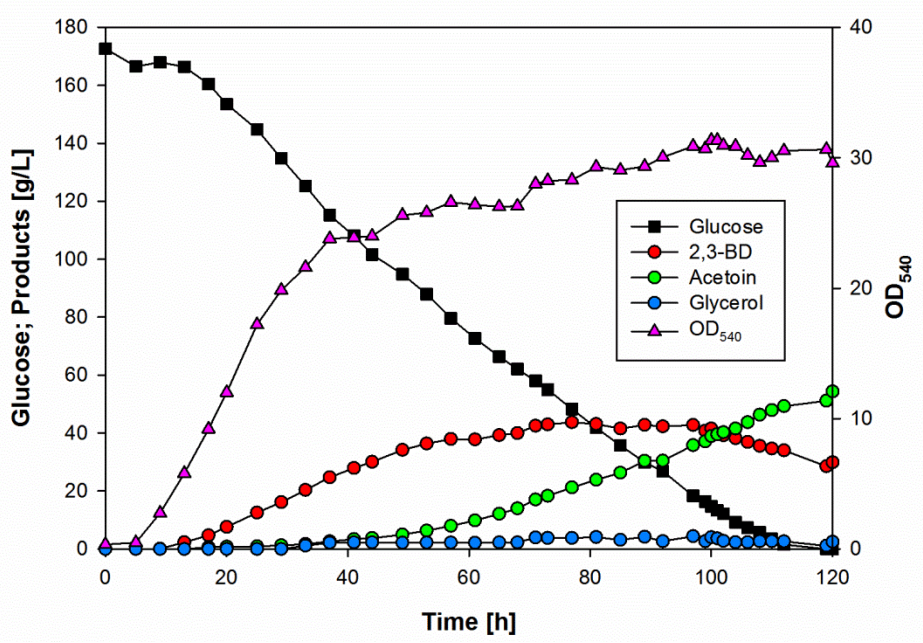

Figure 3-42 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 3.5 L Minifors bioreactor, 2.8 L medium (180 g/L glucose), 200 mL preculture, 30°C, 400 rpm, aeration rate 1.2 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Ortmann, 2012]

After a slow glucose consumption during the first 13 h, the slope of the consumption curve remained constant until 119 h, when no residual glucose remained in the culture. Maximum OD_{540} levels were around 31 after 100 h. The increase in 2,3-BD production was slow, reaching

maximum concentrations of 43.7 g/L after 77 h. Acetoin production started after 37 h and the concentration further increased even after the highest amount of 2,3-BD was reached. The highest amount of acetoin was obtained after 120 h with a value of 54.3 g/L. Glycerol levels were below 5 g/L throughout the entire cultivation.

2,3-BD production on medium with 180 g/L glucose was much lower at 30°C in the 3.5 bioreactor scale compared to the shake flask scale. Therefore, 2,3-BD production at 37°C was investigated; the time course for the corresponding cultivation is illustrated in Fig. 3-43. The glucose concentration present in the medium was completely consumed after 67 h. At the same time point, 78.9 g/L 2,3-BD, 5.1 g/L acetoin and 9 g/L glycerol were detected.

Besides 2,3-BD concentration, yield and productivity were significantly higher at 37°C compared to 30°C, while acetoin production was considerably lower (Tab. 3-27).

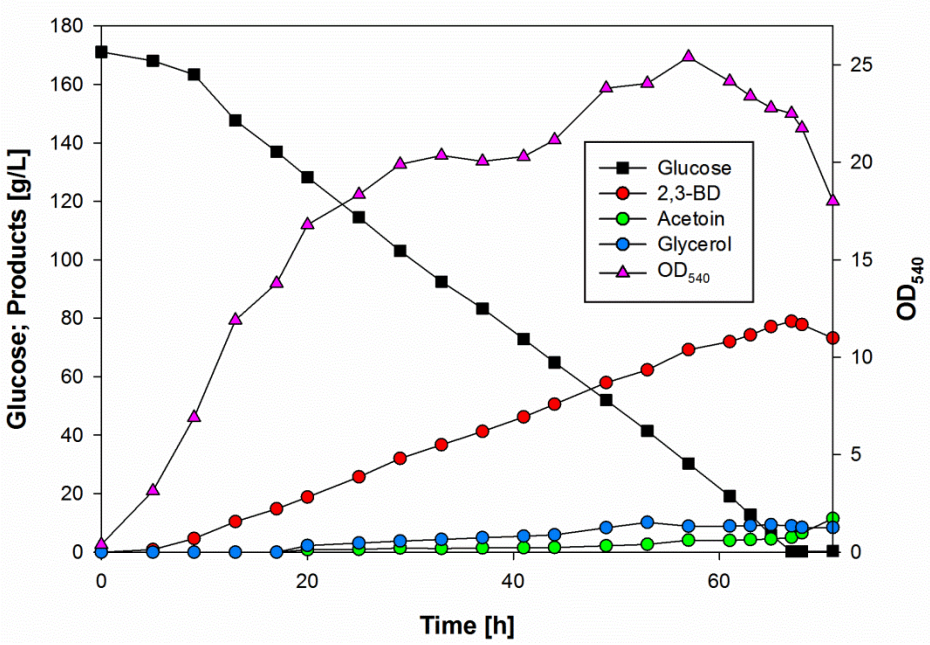

Figure 3-43 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 3.5 L Minifors bioreactor, 2.8 L medium (180 g/L glucose), 200 mL preculture, 37°C, 400 rpm, aeration rate 1.2 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Ortmann, 2012]

Table 3-27 Comparison between bioreactor cultivations of *B. licheniformis* DSM 8785 on medium with 180 g/L glucose at different temperatures [joint work with Ortmann, 2012]

Temperature [°C]	Time [h]	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]	Acetoin [g/L]	Glycerol [g/L]
30°C	77	43.7	0.25	0.57	21.2	3.8
37°C	67	78.9	0.46	1.18	5.1	9.0

3.3.4 Scale-up to the 45 L bioreactor scale on glucose

The scale-up of 2,3-BD production using *B. licheniformis* DSM 8785 from the shake flask scale to the 3.5 L bioreactor scale was successful. Therefore, a further attempt was made to increase the scale up to 45 L (corresponding to 30 L working volume) and to reproduce the results obtained in the 3.5 L bioreactor scale.

3.3.4.1 Scale-up strategies

The first cultivation experiments in the 45 L bioreactor scale were conducted using medium with 120 g/L glucose at 30°C. The dimensions of the two bioreactors used for cultivation experiments are listed in Tab. 3-29.

Table 3-29 Characteristic dimensions of the employed bioreactors

	Abbreviation	3.5 L scale		45 L scale	
Reactor diameter	D	0.138	m	0.31	m
Reactor height	H	0.34	m	0.62	m
Working volume	V	3.0	L	30.0	L
Number of 6-blade disk impellers		2.0		3.0	
Impeller diameter	d	0.054	m	0.125	m

For the scale-up of the aeration rate, a constant superficial gas velocity was employed (Equation 3-1). The aeration rate for the 45 L bioreactor scale was calculated based on the optimized aeration rate of 1.2 L/(L*min) in the 3.5 L scale. The superficial gas velocity v_s corresponds to the ratio between the volumetric gas flow rate and the cross-sectional area of the bioreactor vessel.

$$v_s = \frac{\dot{V}_{(3.5L)}}{\frac{D^2}{4}\pi} = \frac{0.6 \cdot 10^{-5}\ \frac{m^3}{s}}{(0.138m)^2 \cdot \frac{\pi}{4}} = 4.011 \cdot 10^{-3}\ \frac{m}{s} = const. \qquad \textbf{(Equation 3-1)}$$

$$\dot{V}_{(30L)} = v_s \cdot D^2 \cdot \frac{\pi}{4} = 4.011 \cdot 10^{-3}\ \frac{m}{s} \cdot (0.31m)^2 \cdot \frac{\pi}{4} = \qquad \textbf{(Equation 3-2)}$$

$$= 3.027 \cdot 10^{-4}\ \frac{m^3}{s} \approx 0.61 \frac{L}{L \cdot \min} = 18.3 \frac{L}{\min}$$

where: $\dot{V}$ = aeration rate; D = reactor diameter.

According to the equations 3-1 and 3-2, an aeration rate of 18.3 L/min or 0.61 L/(L*min) in the 45 L bioreactor scale corresponds to an aeration rate of 3.6 L/min or 1.2 L/(L*min) in the 3.5 L scale. The scale-up criteria employed in this thesis are the Reynolds number (Re), the impeller tip speed (v_{tip}) and the aeration number (N_B).

(1) Reynolds number

The Reynolds number (Re) is a dimensionless index number illustrating the ratio of inertial force to hydrodynamic viscous forces (Equation 3-3).

$$\mathrm{Re} = \frac{\rho \cdot n \cdot d^2}{\eta} \qquad \textbf{(Equation 3-3)}$$

where: ρ = medium density; η = dynamic viscosity; n = stirring speed; d = impeller diameter.

Considering a constant medium density and a dynamic viscosity, equation 3-3 leads to:

$$n_{30L} \cdot {d_{30L}}^2 = n_{3.5L} \cdot {d_{3.5L}}^2 \qquad \textbf{(Equation 3-4)}$$

$$n_{30L} = \frac{n_{3.5L} \cdot {d_{3.5L}}^2}{{d_{30L}}^2} = \frac{400rpm \cdot (0.054m)^2}{(0.125m)^2} = \textbf{74.65 rpm}. \qquad \textbf{(Equation 3-5)}$$

(2) Impeller tip speed

The impeller tip speed (v_{tip}) is a common scale-up criterion (Hass and Pörtner 2009). The impeller tip speed is a function of the impeller diameter (Equation 3-6).

$$v_{tip} = \pi \cdot d \cdot n \qquad \textbf{(Equation 3-6)}$$

Considering a constant impeller tip speed, equation 3-6 leads to:

$$n_{30L} = \frac{n_{3.5L} \cdot d_{3.5L}}{d_{30L}} = \frac{400rpm \cdot 0.054m}{0.125m} = \textbf{172.8 rpm}. \qquad \textbf{(Equation 3-7)}$$

(3) Aeration number

The aeration number (N_B) is a dimensionless index which corresponds to the ratio of gas input to the stirring rate (Equation 3-8).

$$N_B = \frac{\dot{V}}{n \cdot d^3} \qquad \textbf{(Equation 3-8)}$$

Considering a constant aeration number, equation 3-8 leads to:

$$n_{30L} = \frac{n_{3.5L} \cdot {d_{3.5L}}^3 \cdot \dot{V}_{30L}}{\dot{V}_{3.5L} \cdot {d_{30L}}^3} = \frac{400rpm \cdot (0.054m)^3 \cdot 0.0183 \frac{m^3}{\min}}{0.0036 \frac{m^3}{\min} \cdot (0.125m)^3} = \textbf{163.9 rpm}. \qquad \textbf{(Equation 3-9)}$$

3.3.4.2 Optimization of the impeller speed using different scale-up strategies

Considering that the stirring speed values resulting from the different scale-up criteria lie in the range between 70 and 180 rpm, the first experiment in the 45 L bioreactor scale was carried out using an average stirring speed of 120 rpm. The time course for the corresponding cultivation is displayed in Fig. 3-44. Bacterial growth started directly after inoculation and showed a linear increase during the first 22 h. After 64 h, 0.15 g/L residual glucose were measured, while maximum OD_{540} values around 19 were obtained. At the same time point, 2,3-BD production reached maximum levels of 39.6 g/L. This corresponds to a yield of 0.36 g/g glucose and a productivity of 0.62 g/(L*h). Regarding by-product formation, 2 g/L acetoin and 19.5 g/L glycerol were detected.

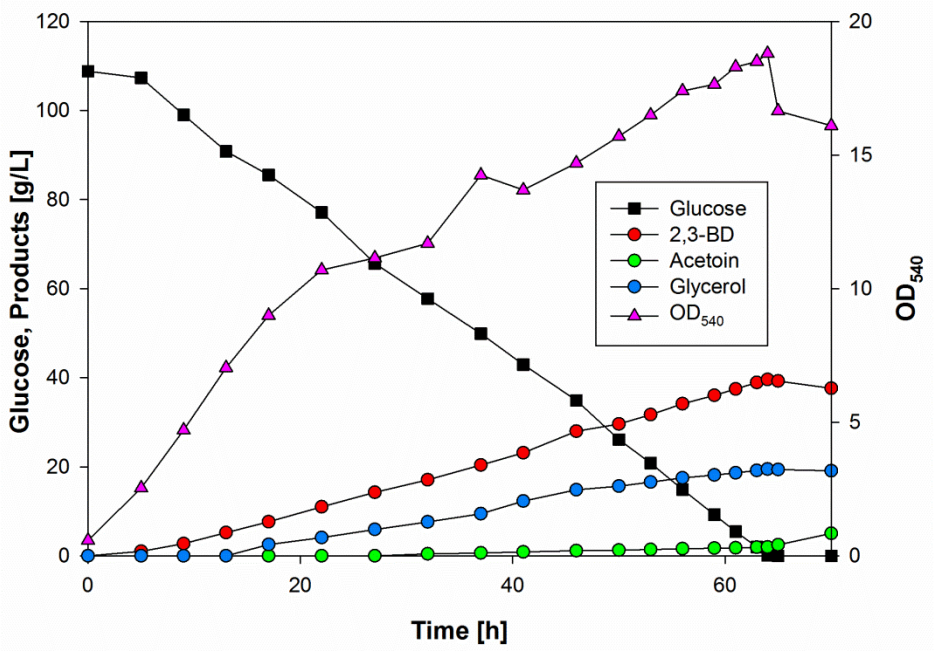

Figure 3-44 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 45 L bioreactor, 28 L medium (120 g/L glucose), 2 L preculture, 30°C, 120 rpm, aeration rate 0.61 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Ortmann, 2012]

For the following cultivation, the aeration number was kept constant. A stirring speed value of 160 rpm was used instead of the calculated value of 163.9 rpm, since the stirring speed could only be set in 10 rpm steps. Fig. 3-45 illustrates the time course for the cultivation performed using a stirring speed of 160 rpm. Glucose consumption was complete after 48.5 h and maximum OD_{540} values of 22 were reached. A maximum 2,3-BD concentration of 42 g/L was obtained after 48 h, corresponding to a yield of 0.39 g/g glucose and a productivity of 0.88 g/(L*h). Glycerol production reached a level of 10.8 g/L, which was lower compared to the cultivation at a stirring speed of 120 rpm. Furthermore, 2.8 g/L acetoin were produced.

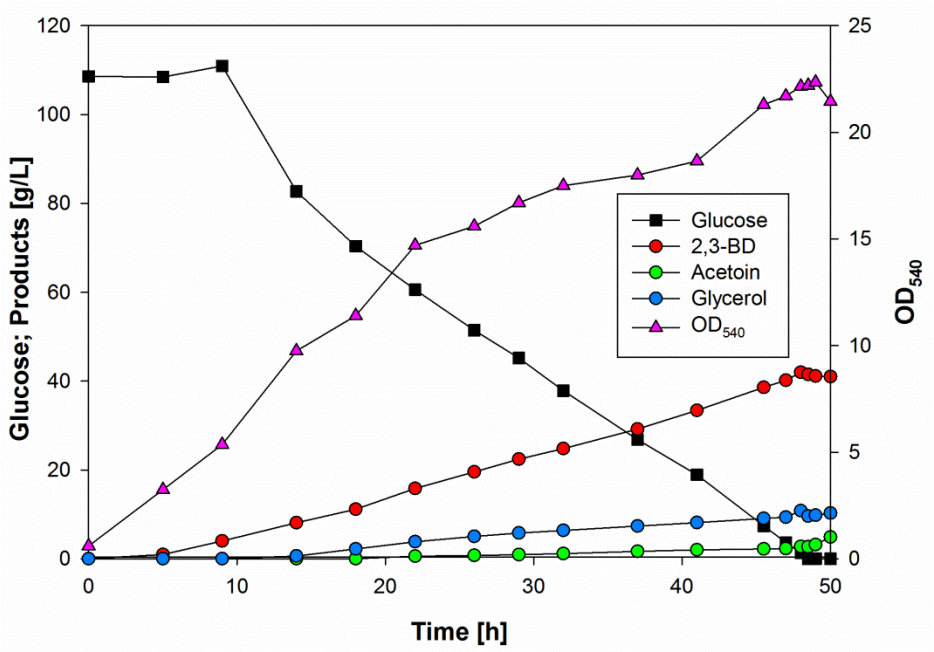

Figure 3-45 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 45 L bioreactor, 28 L medium (120 g/L glucose), 2 L preculture, 30°C, 160 rpm, aeration rate 0.61 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Ortmann, 2012]

Previous experiments showed that glycerol production decreased with a higher oxygen input. Therefore, stirring speed values higher than 160 rpm were tested. Fig. 3-46 shows the time course for the cultivation carried out using a stirring speed of 200 rpm.

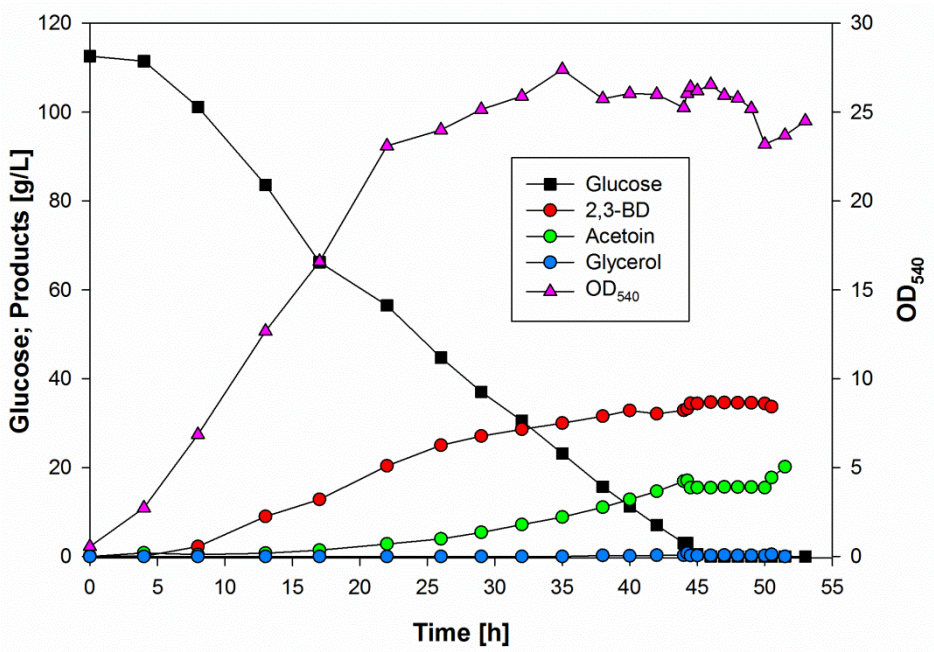

Figure 3-46 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 45 L bioreactor, 28 L medium (120 g/L glucose), 2 L preculture, 30°C, 200 rpm, aeration rate 0.61 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Ortmann, 2012]

Bacterial growth was higher compared to the previous experiments, showing maximum OD_{540} values of 26.6 after 46 h. No residual glucose remained in the medium after 46 h and a maximum amount of 34.7 g/L 2,3-BD was detected. 2,3-BD yield was 0.31 g/g glucose and the productivity 0.76 g/(L*h). Glycerol production remained below 0.5 g/L throughout the entire duration of the cultivation. However, high amounts of acetoin (15.5 g/L) were measured after 44.5 h. Zeng et al. (1994) reported that acetoin can be converted to 2,3-BD, if aeration is switched off. Because of this, aeration was turned off after 44 h at 3 g/L residual glucose. Acetoin concentration decreased and 2,3-BD increased, but only until glucose consumption was complete.

Due to the low yield and productivity as well as the high acetoin production, the stirring speed was reduced to 180 rpm. The time course of the corresponding cultivation is illustrated in Fig. 3-47. Acetoin production was significantly lower, reaching a level of 11.3 g/L after 46 h, when a maximum 2,3-BD concentration of 39.1 g/L was obtained. The yield was 0.36 g/g glucose and the productivity 0.85 g/(L*h). The amount of glycerol was below 2 g/L.

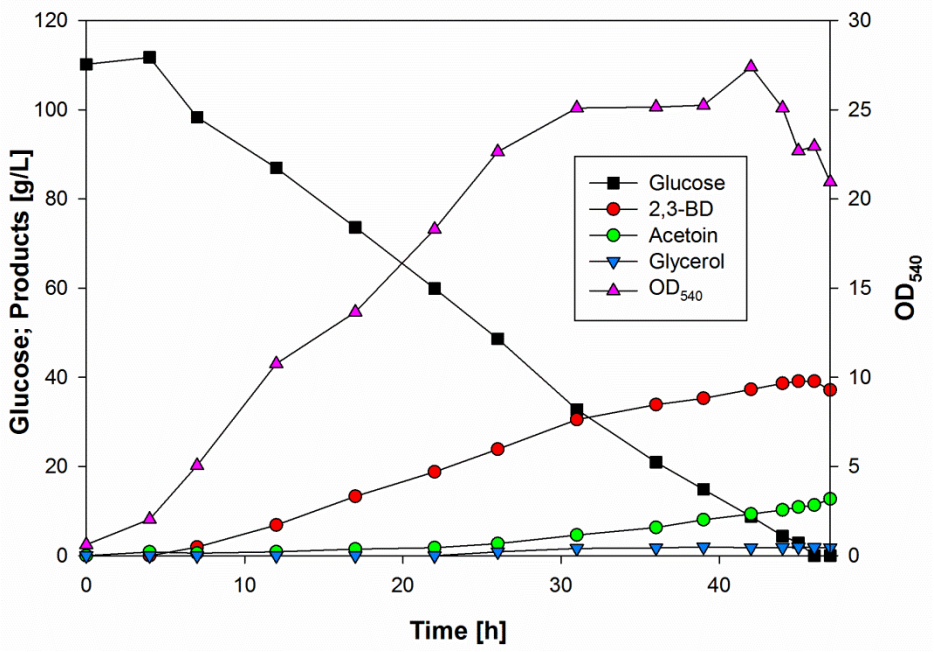

Figure 3-47 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 45 L bioreactor, 28 L medium (120 g/L glucose), 2 L preculture, 30°C, 180 rpm, aeration rate 0.61 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Ortmann, 2012]

Additionally, a cultivation experiment with a stirring speed of 170 rpm was carried out, which corresponds to using the impeller tip speed as scale-up criterion (Fig. 3-48). The highest 2,3-BD production was measured after 45 h with a concentration of 41.6 g/L. This corresponds to a yield of 0.39 g/g glucose and a productivity of 0.92 g/(L*h). Regarding by-product formation, 5.7 g/L acetoin and 7.6 g/L glycerol were measured after 45 h.

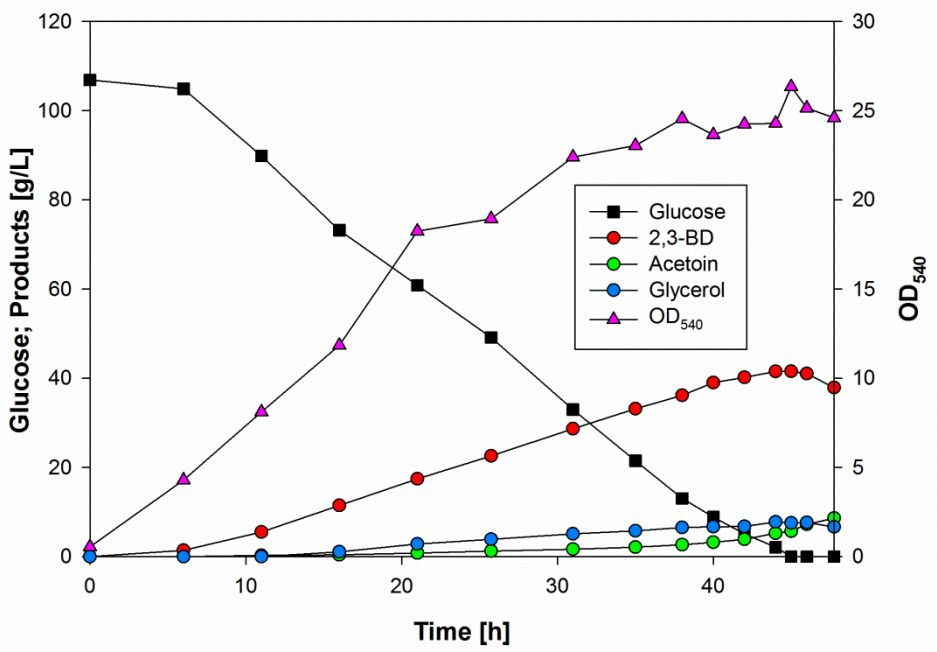

Figure 3-48 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 45 L bioreactor, 28 L medium (120 g/L glucose), 2 L preculture, 30°C, 170 rpm, aeration rate 0.61 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Ortmann, 2012]

Tab. 3-30 summarizes the results of the scale-up experiments from the 3.5 L to the 45 L bioreactor scale using *B. licheniformis* DSM 8785. The highest productivity was reached with a stirring speed of 170 rpm. The yield was equal for stirring speed values of 160 and 170 rpm. The highest 2,3-BD concentration was in the same range during cultivation with 160 or 170 rpm. With increasing stirring speed, an increase in acetoin concentration and a decrease in glycerol production could be detected.

Table 3-30 Comparison of the results of scale-up experiments using *B. licheniformis* DSM 8785 at 30°C on medium with 120 g/L glucose [joint work with Ortmann, 2012]

Conditions	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]	Acetoin [g/L]	Glycerol [g/L]
45 L scale					
120 rpm	39.6	0.36	0.62	2.0	19.5
160 rpm	**42.0**	**0.39**	0.88	2.8	10.8
170 rpm	**41.6**	**0.39**	**0.92**	5.7	7.6
180 rpm	39.1	0.36	0.85	11.3	1.8
200 rpm	34.7	0.31	0.76	15.5	0.2
3 L scale					
400 rpm	47.6	0.43	0.85	7.1	4.3

3.3.4.3 Scale-up at higher initial glucose concentrations (180 g/L)

The results of scale-up experiments conducted using medium with 120 g/L glucose were presented in chapter 3.3.4.2. Further cultivation experiments were performed on medium with 180 g/L glucose at 37°C in the shake flask and 3.5 L bioreactor scale. The results of these cultivations were shown in chapter 3.3.1.2 (Fig. 3-27) and chapter 3.3.3.6 (Fig. 3-43 and Tab. 3-27), respectively. In the shake flask scale 70.1 g/L 2,3-BD, a yield of 0.39 g/g glucose and a productivity of 0.95 g/(L*h) were obtained. The cultivation in the 3.5 L bioreactor scale led to a 2,3-BD production of 78.9 g/L, a yield of 0.46 g/g glucose and a productivity of 1.18 g/(L*h).

Based on these results, a scale-up of 2,3-BD production to the 45 L bioreactor scale at 37°C using medium with 180 g/L glucose was carried out. An aeration rate of 0.61 L/(L*min) (18.3 L/min) and a stirring speed of 170 rpm were employed. Fig. 3-49 illustrates the time course for this cultivation. The initial glucose concentration was 153.7 g/L, due to the fact that the glucose solution for the medium was prepared directly in the bioreactor, so the volume could not be accurately adjusted. The maximum 2,3-BD production reached 65.1 g/L after 54 h, which is lower than the concentrations obtained in the smaller scales. However, the yield of 0.42 g/g glucose and the productivity of 1.19 g/(L*h) were higher than in the smaller scales. Maximum OD_{540} values of 19.5 were measured after 38-52 h. The amount of by-products detected after 54 h were 4.6 g/L acetoin and 13.7 g/L glycerol.

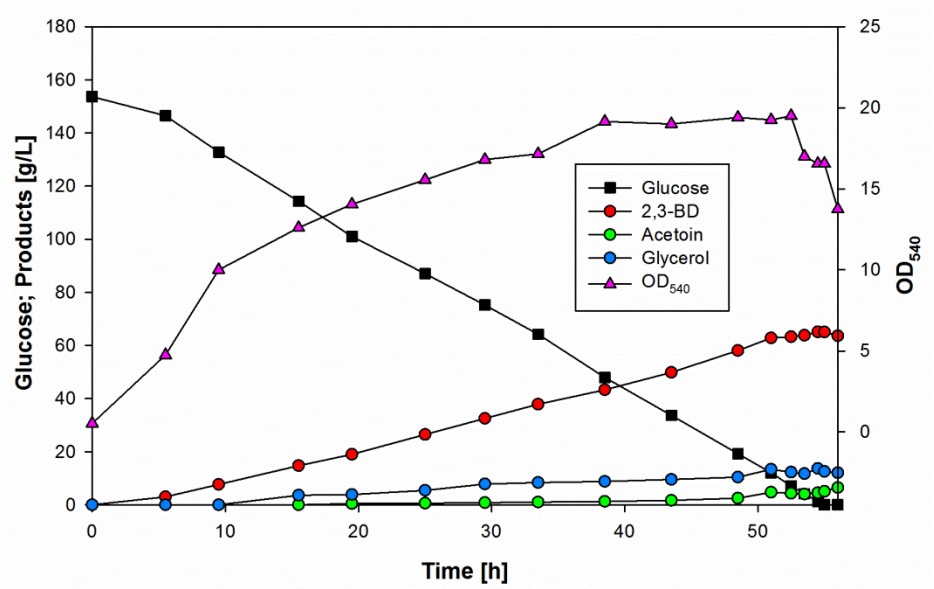

Figure 3-49 Time course for the batch cultivation of *B. licheniformis* DSM 8785. Conditions: 45 L bioreactor, 28 L medium (180 g/L glucose), 2 L preculture, 37°C, 170 rpm, aeration rate 0.61 L/(L*min), initial pH 6.6 (not adjusted) [joint work with Ortmann, 2012]

A comparison of glucose consumption and 2,3-BD production during cultivation with *B. licheniformis* DSM 8785 at 37°C using an initial glucose concentration of 180 g/L in the shake flask scale as well as the 3.5 L and 45 L bioreactor scale is shown in Fig. 3-50. The main results of these experiments are summarized in Tab. 3-31.

2,3-BD production was much slower in the shake flask scale, while the production rate in the 3.5 L and 45 L bioreactor scale were almost identical. The productivities were therefore in the same range for the two bioreactor scales (Tab. 3-31). However, the maximum amount of 2,3-BD was lower in the 45 L bioreactor scale, corresponding to the lower initial glucose amount. Glucose consumption rate and acetoin production were similar in all three scales. However, glycerol concentrations were significantly higher in the shake flask scale, leading to a lower 2,3-BD yield. The lowest glycerol production and highest 2,3-BD yield were detected in the 3.5 L bioreactor scale.

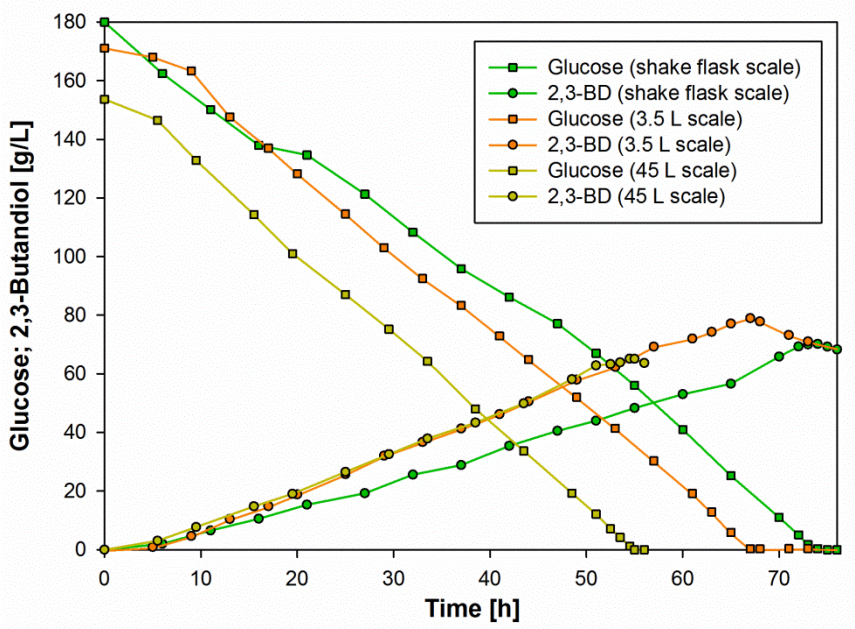

Figure 3-50 Comparison between glucose consumption and 2,3-BD production on medium with 180 g/L glucose using *B. licheniformis* DSM 8785 in three different scales (shake flask, 3.5 L bioreactor, 45 L bioreactor) [joint work with Ortmann, 2012]

Table 3-31 Comparison of the results of cultivations using *B. licheniformis* DSM 8785 at 37°C on medium with 180 g/L glucose in the different scales [joint work with Ortmann, 2012]

Scale	Max. 2,3-BD [g/L]	Yield [g/g]	Productivity [g/(L*h)]	Acetoin [g/L]	Glycerol [g/L]
Shake flask	70.1	0.39	0.95	5.0	21.5
3.5 L bioreactor	78.9	0.46	1.18	5.1	9.0
45 L bioreactor	65.1	0.42	1.19	4.6	13.7

4 Discussion

Research has been conducted on microbial 2,3-BD synthesis since 1906, when *Klebsiella pneumoniae* was successfully employed for the production of the diol in the laboratory scale (Harden and Walpole 1906) and also the industrial scale (Fulmer et al. 1933). Since then, numerous efforts have been made in searching for better performing microbial strains and improving fermentation processes.

In recent years, due to the shortage of crude oil reservoirs and rising petroleum prices, the microbial production of 2,3-BD based on renewable raw materials has increasingly gained significance (Hatti-Kaul et al. 2007; Celińska and Grajek 2009; Ji et al. 2011). Moreover, the use of low-cost renewable feedstocks, such as residues from agricultural, forestry and food processing industry, would reduce the cost for an industrial fermentation process (Celińska and Grajek 2009; Zeng and Sabra 2011). An additional cost reducing factor would be the use of risk group 1 (non-pathogenic) microorganisms (e.g. *Paenibacillus polymyxa*, *Bacillus* sp.).

The objective of the PUBB project was the development of an efficient fermentative process for 2,3-BD production from low-cost renewable feedstocks using non-pathogenic microorganisms. In order for this process to be efficient compared to employing risk group 2 strains, maximum 2,3-BD production, yield and productivity have to be improved using non-pathogenic bacteria.

For this reason, after screening for suitable non-pathogenic strains for 2,3-BD production, the medium composition and fermentation conditions should be optimized. Afterwards, with optimized parameters a scale-up of 2,3-BD production from the shake flask scale to the 3.5 L and even up to the 45 L bioreactor scale should be implemented.

In chapter 3 the results of these experiments have been presented. In the present chapter, these results will be discussed and compared to results from literature.

4.1 Screening for 2,3-BD producing microorganisms

4.1.1 Wild type strains from culture collections

Several non-pathogenic microorganisms obtained from culture collections and project partners were investigated on their potential to produce 2,3-BD. The first cultivation experiments were carried out in the shake flask scale on medium containing 30 g/L glucose and the resulting maximum 2,3-BD concentrations were compared. The best results (approx. 12-15 g/L 2,3-BD) were obtained with *P. polymyxa* ATCC 12321, DSM 356 and DSM 36 as well as *B. licheniformis* DSM 8785 and ATCC 9879. As reference, risk group 2 strains *K. oxytoca* ATCC 8724, NRRL B-199 and m5a were employed and yielded 11.5-13 g/L 2,3-BD.

The best producing strains were cultivated on natural wood hydrolysate medium containing 30 g/L glucose. Risk group 2 *K. oxytoca* produced 8.8-12.6 g/L 2,3-BD, while with the three *P. polymyxa* strains and *B. licheniformis* DSM 8785 amounts of 10.5-14.3 g/L 2,3-BD could be detected. With *B. licheniformis* ATCC 9879 2,3-BD synthesis on natural wood hydrolysate was not successful, since amounts lower than 1 g/L were reached.

The four best strains were further tested on glucose medium with increased glucose concentration (60 g/L). *P. polymyxa* ATCC 12321 and DSM 356 and *B. licheniformis* DSM 8785 produced 21.8-23.5 g/L 2,3-BD and yields between 0.36-0.39 g/g glucose. Regarding the obtained productivities, only *P. polymyxa* ATCC 12321 and *B. licheniformis* DSM 8785 showed high values of 0.74 and 0.87 g/(L*h), respectively. Hence, these two strains were employed for further optimization processes.

4.1.2 Strain improvement by random mutagenesis

The two best strains for 2,3-BD production selected after the screening step according to the results presented in chapter 3.1.1 were further subjected to random mutagenesis using UV light (260 nm).

After UV irradiation, potential mutants of *P. polymyxa* ATCC12321 were cultured in 96-well plates. 44 potential mutants showing low color shift (corresponding to low acid production) were isolated and subjected to TLC analysis. For 6 of the tested mutants no 2,3-BD production could be detected. The other 38 potential mutants were cultivated in the shake flask scale on glucose medium. However, none of the tested mutants were able to produce higher amounts of 2,3-BD compared to the wild type strain after 10 h of cultivation. A possible explanation for this may be the longer lag phase for the cultivation of potential mutants. A longer cultivation time would therefore be required for the potential mutants to reach maximum 2,3-BD amounts comparable to the wild type strain. This would however lead to lower productivities.

From the 59 potential mutants of *B. licheniformis* DSM 8785 selected after cultivation in 96-well plates, 20 mutants were selected based on positive 2,3-BD production by TLC. The results of the cultivation of these mutants in the shake flask scale showed that mutants no. 35 and no.36 did not grow and therefore were not able to produce 2,3-BD. There was no significant difference between the highest 2,3-BD amount obtained using the wild type strain (47.3 g/L) and the other 18 mutants (46.2-51 g/L). This slight increase in 2,3-BD production is probably not significant for an industrial fermentation process. Furthermore, productivities obtained during cultivation with mutants were significantly lower, due to the longer cultivation time (40-48 h) compared to the wild type strain (36 h).

Unfortunately, the results regarding strain improvement using random mutagenesis obtained by Ji et al. (2008) could not be reproduced for *P. polymyxa* ATCC 12321 and *B. licheniformis* DSM 8785. However, in the aforementioned study, random mutagenesis using UV coupled with diethyl sulfate (DES) and a modified proton suicide selection strategy were employed for *K. oxytoca* ME-303.

Further possibilities would be to repeat the experiment and isolate more potential mutants, thus increasing the possibility of finding a mutant with better 2,3-BD producing capability.

4.1.3 Isolation of microorganisms from natural habitats

Microbial strains suitable for 2,3-BD production were also isolated from environmental samples. From water, driftwood and addled wood samples collected at the Steinhuder Sea 12 strains were isolated on selective agar plates.

Cultivation experiments on glucose medium showed that only 6 of the tested strains were able to produce 2,3-BD. Only 2 of these microorganisms were found to belong to the risk group 1 after partial 16S rDNA-sequencing.

2,3-BD production of the 2 isolates on wood hydrolysate medium was however lower compared to *P. polymyxa* ATCC 12321 and *B. licheniformis* DSM 8785 wild type strains. The same behavior could be observed for the yield and productivity of the isolates. As a result, the isolated strains were not considered for further investigations.

4.2 2,3-BD production with *Paenibacillus polymyxa* ATCC 12321

4.2.1 Shake flask experiments on glucose

Batch experiments performed with free cells of *P. polymyxa* ATCC 12321 indicated that an initial glucose concentration of around 60 g/L is optimal for high 2,3-BD production. Preliminary cultivation tests were performed on medium with 20-80 g/L glucose in order to determine a reasonable "study area" for the CCD optimization. A maximum of 22.6 g/L 2,3-BD was reached from 60 g/L glucose. A further increase in glucose concentration from 60 to 80 g/L led to a slight decrease in 2,3-BD synthesis. Additionally, the amount of residual glucose increased from levels below 2 g/L to 22.8 g/L. These results led to choosing glucose concentrations of 50, 70 and 90 g/L for the -1, 0 and +1 levels of the study area for CCD optimization. For the temperature, the lowest level of 25°C was based on data from literature, while the highest level of 35°C was chosen considering the increased evaporation at higher temperatures. Regarding the shaking rate, the value of 50 rpm for the -1 level was selected based on future scale-up experiments to the bioreactor scale, where cultivations without stirring are not reasonable. For the +1 level a shaking rate of 150 rpm was chosen, considering that at higher values, the liquid contained in the shake flasks would reach the cotton plug, hereby increasing the risk for microbial contamination.

By using the CCD optimization with respect to high 2,3-BD production, the best conditions were computed as follows: 68 g/L glucose, 25°C and 80 rpm. With regard to obtaining a high productivity, optimum computed parameters were: 50 g/L glucose, 35°C and 150 rpm. The computed maximum 2,3-BD concentration of 23.4 g/L could be confirmed by performing the actual cultivation, which led to 24.4 g/L 2,3-BD. The computed productivity was 1.4 g/(L*h), the actual value measured during cultivation 1.5 g/(L*h).

On the subject of comparing the use of yeast extract and tryptone or urea as nitrogen source, results showed that the mixture of yeast extract / tryptone led to the production of 12 g/L 2,3-BD after 30 h. The cultivation employing urea as nitrogen source was unsuccessful, enabling only a poor glucose consumption and the synthesis of less than 1 g/L 2,3-BD. Other alternative nitrogen sources should be tested for their suitability to replace the complex supplements or a microbial adaptation on medium containing urea should be performed in order to improve the utilization of this compound.

For the cultivation using immobilized cells of *P. polymyxa* ATCC 12321 in form of LentiKats® the parameters for maximum 2,3-BD production were employed. The experiment was carried out as a repeated-batch cultivation, consisting of 3 batch phases and medium replacements in between. During the first batch phase, the immobilized cells were cultivated for 140 h, but the

entire glucose was already completely consumed after 57 h. The maximum 2,3-BD concentration of 31.1 g/L reached after 57 h started to decrease as soon as no glucose was left in the medium. This can be explained by the fact that in the absence of glucose, the bacteria found in 2,3-BD an alternative carbon source and gained their required energy by converting 2,3-BD to acetoin. This assumption is supported by the significant increase in acetoin concentration, which corresponded to the decrease in 2,3-BD levels.

After the first medium replacement, the cultivation was carried out for only 50 h in order to avoid a complete depletion of glucose. The 2,3-BD concentration reached levels of 31.2 g/L before and 31.9 g/L 60 h after the second medium exchange was carried out. After each medium replacement an equally high 2,3-BD amount could be obtained. The measured concentrations were even higher compared to the results obtained using free cells, where 24.4 g/L 2,3-BD were reached (see chapter 3.2.1.5, Tab. 3-7). A possible explanation could be found by comparing the amount of by-products produced during cultivation with free cells to those obtained from immobilized cells. The results from the experiment conducted with free cells led to the synthesis of 6.3 g/L acetoin and 5.1 g/L ethanol. During the cultivation using LentiKats®, 1.1 g/L acetoin and 6.4 g/L ethanol were detected. The increased amount of 2,3-BD obtained with immobilized cells corresponded to the decreased concentration of acetoin. The shorter cultivation time required to reach maximum 2,3-BD amount and corresponding higher productivity during LentiKats® cultivation is probably due to the up to 2.5-fold increased amount of cells employed compared to the experiment using free cells.

When taking a look at the OD_{540} values corresponding to the growth outside the LentiKat® particles, it is obvious that these values are significantly higher after the two medium replacements. This almost up to 4-fold increase can be explained by a possibly incomplete removal of cells during the washing steps with 0.1 M magnesium sulfate solution, which would allow the microorganisms to replicate faster after the addition of fresh medium to the shake flasks.

Three positive effects were observed during cultivation experiments using LentiKats®. First of all, the cells remained vital and active even 83 h after glucose depletion and were able to resume 2,3-BD production after medium replacement. Secondly, the cultivation period until maximum 2,3-BD concentration was reached was shorter compared to experiments using free cells. Finally, higher product concentrations could be obtained by employing LentiKats®.

In consideration of a future industrial 2,3-BD production, experiments were carried out using the fed-batch cultivation mode in order to reach a maximum 2,3-BD concentration in a short time. Therefore, the optimal parameters for high productivity were selected for the fed-batch experiments. These parameters ensured a high productivity and maximum 2,3-BD amount

after only 10 h of cultivation. In order to avoid a metabolic shift caused by glucose depletion and a consumption of the desired product, feeding steps were carried out every 10 h. For each feeding, 5 g glucose and corresponding amounts of the other nutrients were added in solid form to the culture. This way, the glucose concentration was increased up to 50 g/L with each feeding step. The maximum 2,3-BD concentration could be enhanced up to a level of 63.3 g/L after the second feeding step. The yield was 0.42 g/g glucose, while the productivity increased up to 2.26 g/(L*h). The concentration of by-products (acetoin and ethanol) remained below 10 g/L. The decline in microbial growth after 26 h, which continued after the third feeding step could be a sign of an initiating product inhibition or of the low water activity in the culture due to repeated feeding of solid compounds.

4.2.2 Shake flask experiments on wood hydrolysates

The comparison between 2,3-BD production by *P. polymyxa* ATCC 12321 from natural and artificial wood hydrolysate with 30 g/L glucose revealed similar results for both cultivation experiments. Moreover, both yield and productivity values were higher for the cultivation on natural wood hydrolysate medium.

The effect of the potential inhibitory compounds furfural, 5-HMF, 5-hydroxybenzoic acid, vanillin, syringaldehyde, formic acid and acetic acid on glucose consumption and 2,3-BD production by *P. polymyxa* ATCC 12321 was investigated using different concentration ranges. During cultivation on wood hydrolysates, the potential inhibitory compounds are metabolized in different ways.

Furfural is reduced to furfuryl alcohol, while under aerobic conditions it can also be oxidized to furoic acid (Palmqvist et al. 1999; Palmqvist and Hahn-Hägerdal 2000). Suggested inhibition mechanisms of furfural are the interference with electron transport, the inhibition of glycolytic enzymes and the depletion of NAD(P)H were found in *S. cerevisiae* (Vitrinskaya and Soboleva 1975; Barenjee et al. 1981; Almeida et al. 2007). In *S. cerevisiae*, HMF is converted to 5-hydroxymethyl furfuryl alcohol and suggested inhibition mechansims are similar to those of furfural (Taherzadeh et al. 2000). HMF metabolization was reported to occur at a lower rate compared to furfural, probably due to lower membrane permeability and therefore causing a longer growth lag-phase (Larsson et al. 1999).

Phenolic compounds have been found to inhibit fermentations by damaging the biological membranes, thereby impairing their ability to serve as selective barriers and enzyme matrices (Heipieper et al. 1994). An inhibitory effect of concentrations of 1 g/L of 4-hydroxybenzoic acid and vanillin during cultivations with *S. cerevisiae* was reported by Ando et al. (1986).

Weak acids inhibit cell growth by two possible mechanisms: uncoupling and intracellular anion accumulation (Russel 1992). The uncoupling theory states that in the presence of weak acids the decrease in intracellular pH is counterbalanced by the plasma membrane ATPase, which pumps protons out of the cell at the expense of ATP hydrolysis (Verduyn et al. 1992). High concentrations of acid lead to the exhaustion of the proton pumping ability of the cell and the depletion of the ATP supply (Imai and Ohono 1995). The intracellular anion accumulation theory states that the anionic form of acids is captured in the cell; in order to reach a state of equilibrium, undissociated acid diffuses into the cell. Furthermore, the pH gradient over the plasma membrane will influence the degree of intracellular anion accumulation (Russel 1992; Almeida et al. 2007).

According to the results obtained during cultivation in the presence of single inhibitory compounds, no inhibitory effect could be detected using a 1-fold concentration of most tested compounds with the exception of 4-hydroxybenzoic acid. Regarding the cultivation with addition of this compound, even the lowest concentration (0.169 g/L) required 5 h extra time for maximum 2,3-BD concentration to be reached. With increasing amount of 4-hydroxybenzoic acid, the glucose consumption and 2,3-BD production were progressively slower. In the case of acetic acid, a time lag could be observed from a 16-fold concentration of the compound (24 g/L). Moreover, lower amounts of acetic acid showed a positive effect on 2,3-BD production. Concentrations of 1.5 g/L, 3 g/L and 6 g/L (corresponding to a 1-fold, 2-fold and 4-fold amount) were proven to accelerate 2,3-BD synthesis by 5-13 h. An 8-fold concentration of acetic acid (12 g/L) revealed no acceleration of product synthesis, but 5 g/L more 2,3-BD were detected in the culture broth. This confirms the report of Nakashimada et al. (2000), according to which an enhancement of 2,3-BD production can be obtained by addition of up to 150 mM (12 g/L) acetate.

Concerning the experiments performed by adding a combination of all tested compounds, no inhibitory effect could be observed at the original (1-fold) concentration. Furthermore, an acceleration of 2,3-BD production was ensured by the acetate added to the culture. This compensates for the inhibiting effect of 4-hydroxybenzoic acid. An inhibiting effect on bacterial growth could be detected from an 8-fold amount of combined inhibitors. In this case, the positive effect of acetate addition is overcompensated by the inhibiting effect of the other compounds.

For further batch cultivations on natural wood hydrolysate with 68 g/L glucose, no inhibition by the present furans, phenolic compounds and organic acids should be expected. However, the critical concentration of inhibitors might be exceeded during fed-batch cultivation experiments, depending on the amount of nutrients and frequency of the feedings.

4.2.3 Scale-up to the 3.5 L bioreactor on artificial wood hydrolysates

In contrast to shake flask cultivation experiments, a temperature of 30°C was found to be more suitable for 2,3-BD production with *P. polymyxa* ATCC 12321 in the 3.5 L bioreactor scale compared to 25°C. Glucose consumption and 2,3-BD synthesis were accelerated for 10 h by the increase in incubation temperature, which led to an enhancement in productivity.

With regard to the optimization of the aeration rate, a value of 1.2 L/(L·min) was found to be optimal for 2,3-BD production in the 3.5 L bioreactor. Maximum 2,3-BD concentration was reached after 56 h when the cultivation was conducted at 25°C using an aeration rate of 0.8 L/(L·min). An aeration rate of 1.2 L/(L·min) led to a maximum product amount after only 48 h. Additionally, 1 g/L less 2,3-BD as well as higher by-product concentrations were obtained at 0.8 L/(L·min) and 25°C. The average RQ value of 1 points to a metabolic shift towards the citric acid cycle (results not shown). A higher aeration rate of 1.5 L/(L·min) was unsuitable for 2,3-BD production. Glucose consumption and 2,3-BD production occurred slower compared to the lower aeration rates. After 35 h an almost complete cellular lysis occurred, which can be seen by the abrupt decrease in OD_{540} values. In addition, glucose consumption ceased and a residual amount of 9 g/L remained in the culture broth.

2,3-BD concentrations obtained in the shake flask scale were successfully reproduced in the 3.5 L bioreactor scale. Concerning by-product formation, during cultivations in the 3.5 L bioreactor scale glycerol was obtained as by-product at 25°C, while lactate was detected during cultivations performed at 30°C. Glycerol production at 25°C could be considered as a stress reaction due to a lower temperature, since its synthesis could be detected at 30°C.

The aim of bioreactor cultivations with forced pH shifts was an improved productivity, which was obtained with two of the tested pH shifts (5.3 → 5.8 and 5.8 → 6.3). The highest productivity, 0.80 g/(L*h) resulted from the cultivation performed with a forced pH shift from 5.3 → 5.8. Petrov and Petrova (2010) reported obtaining best results regarding 2,3-BD production when cultivations with *K. pneumoniae* G31 and forced pH fluctuations of 1 pH unit were carried out in 12 h time intervals. However, best productivities were reported when pH fluctuations of 3 pH units were conducted in 24 h intervals. But the obtained value of 0.57 g/(L*h) was significantly lower than the productivities obtained in this thesis with forced pH shifts. A further positive effect on 2,3-BD production could, however, not be detected. *P. polymyxa* ATCC 12321 seemed to react not only on the actual pH value, but also on its time course during cultivation. The higher pH set through the forced fluctuations from 5.8 → 6.3 and 5.3 → 6.3 led to an increased lactate production from glucose to the detriment of 2,3-BD synthesis. It could be noticed, that 2,3-BD production was induced by the drop in pH. When the pH reached a plateau, 2,3-BD synthesis ceased and acetoin production intensified.

An increase in 2,3-BD production in the 3.5 L bioreactor scale could be achieved by using the fed-batch cultivation mode. A maximum 2,3-BD amount of 44.5 g/L was reached. Moreover, an increased yield of 0.43 g/g was obtained in comparison to the yield of 0.37 g/g detected during batch cultivations in the 3.5 L bioreactor scale. The productivity of 0.57 g/(L*h) was slightly lower compared to the batch cultivation mode. After the first 2 feedings, a significant increase in 2,3-BD production could be detected. However, the feeding induced a pH shift, which could be the reason for the enhancement in 2,3-BD synthesis. After 30 h, 2,3-BD production stagnated and acetoin production initiated. During this time, the pH remained more or less constant. This effect could be avoided by performing a feeding step at 40 g/L residual glucose. The third feeding did not contribute to a further enhancement of 2,3-BD synthesis. A metabolic shift could be a possible explanation considering the significant increase in acetoin synthesis, while microbial contamination should also not be disregarded. The supplementation of solid non-sterilized compounds to the culture broth presents a high contamination risk. The results obtained in the bioreactor scale were, however, lower than those obtained in the shake flask scale. A possible reason is the higher temperature of 35°C employed in the shake flask scale, which could have positively affected the productivity. Furthermore, glucose concentrations were maintained below 55 g/L by the feedings during shake flask cultivations. These effects should be investigated during further experiments.

4.2.4 Conclusions on 2,3-BD production with *P. polymyxa* ATCC 12321

Shake flask cultivations showed that a glucose concentration of 68 g/L, 25°C and 80 rpm are optimal conditions for high 2,3-BD production with *P. polymyxa* ATCC 12321, while a glucose amount of 50 g/L, 35°C and 150 rpm lead to high productivities. The LentiKats® immobilization technique was proven successful for high 2,3-BD production, showing high viability and activity after several medium replacements. Moreover, using the fed-batch cultivation mode, 2,3-BD production could be increased up to 63.3 g/L and the productivity up to 2.26 g/(L*h).

Cultivations on natural wood hydrolysates were similar to corresponding experiments on glucose medium or artificial wood hydrolysates. The amount of potential inhibitory compounds present in natural wood hydrolysates with 68 g/L glucose showed no negative effect on bacterial growth and 2,3-BD production.

Regarding cultivations performed in the 3.5 L bioreactor scale, 30°C, 400 rpm and an aeration rate of 1.2 L/(L*min) were detected as optimal conditions for a successful scale-up. Furthermore, using forced pH fluctuations from 5.3 → 5.8, an increase in productivity could be obtained.

4.3 2,3-BD production with *Bacillus licheniformis* DSM 8785

4.3.1 Shake flask experiments on pure sugars

Batch experiments performed with free cells of *B. licheniformis* DSM 8785 indicated that 2,3-BD production is influenced by the amount of glucose added to the culture medium. Starting with an amount of glucose of 20 g/L, each increase in the initial glucose concentration (up to 180 g/L) resulted in a higher production of 2,3-BD.

Furthermore, it can be noted that the increase in 2,3-BD synthesis is proportional to the increase in the amount of glucose present in the medium. 2,3-BD yields therefore lie in the same range (0.38 - 0.42 g/g glucose) for glucose concentrations between 60 and 200 g/L. The theoretical yield of 2,3-BD production from glucose is 0.5 g/g used sugar (Voloch et al. 1985). The highest yield obtained in batch experiments with free cells (0.42 g/g glucose from 180 g/L glucose) corresponds to 84% of the theoretical yield. Nilegaonkar et al. (1992) reported a maximum yield of 0.47 g/g glucose, obtained during cultivation with *B. licheniformis* on medium with 20 g/L glucose. This value is higher than all obtained yields in this thesis, which is probably due to the higher concentration of beef extract (10% w/v) employed in the mentioned study. The highest productivity reported in the above mentioned study was 0.12 g/(L*h), which is significantly lower than the values reached in this thesis with *B. licheniformis* DSM 8785. The highest productivity obtained in the present study was 0.99 g/(L*h), reached on medium with 120 g/L glucose.

An increase in glucose concentration above 180 g/L led to a reduction in maximum 2,3-BD levels as well as higher residual glucose concentrations. An initial glucose concentration of 180 g/L was proven to be optimal for 2,3-BD production using *B. licheniformis* DSM 8785 on medium with 5 g/L yeast extract and 5 g/L tryptone. This amount of glucose in the culture medium led not only to the highest yield, but also to the highest level of 2,3-BD obtained with this strain in batch experiments (72.6 g/L).

Using the fed-batch cultivation mode, 2,3-BD production using *B. licheniformis* DSM 8785 could be further increased. With an optimum initial glucose concentration of 180 g/L and feeding of additional 180 g/L glucose and other nutrients (in two steps) a maximum 2,3-BD concentration of 144.7 g/L could be obtained. The yield (g/g glucose) was similar to the batch experiments, while a significant increase in the volumetric productivity (from 0.86 to 1.14 g/(L*h)) could be noted. This means that increasing the concentration of sugar and other nutrients during the process, when cell growth is strong and cells are most active may be more favorable compared to a higher initial sugar concentration. This way the cells are not subjected directly at the beginning of the cultivation to high glucose concentrations when inhibition

phenomena can occur, but are kept active by stepwise feeding of nutrients. This is a possibility to further increase product concentrations by-passing a limitation concerning the initial sugar concentration.

Concerning 2,3-BD production from sucrose, cultivation results showed that 2,3-BD concentration increased with a higher amount of initial sucrose in the medium. Furthermore, compared to experiments on glucose, an increase in product concentration was observed up to an initial concentration of sucrose of 260 g/L. However, the yields were significantly lower from sucrose (0.31-0.32 g/g) compared to cultivations on glucose (0.40-0.42 g/g). The productivities were in the same range for both sugars.

Cultivation temperatures higher than 30°C showed no positive effect on 2,3-BD production by *B. licheniformis* on medium with 120 g/L glucose. Maximum product levels and yields were in the same range, while the productivity decreased at 35°C. However, for cultivations on medium with a higher initial glucose concentration (180 g/L), an increase in incubation temperature from 30 to 37°C resulted in an increase in productivity from 0.86 g/(L*h) to 0.95 g/(L*h), while the values of 2,3-BD amount and yield were similar for both temperatures.

Regarding the comparison between using a mixture of yeast extract and tryptone or urea as nitrogen source, the best results were obtained from cultivations on yeast extract / tryptone. Maximum 2,3-BD production, yield and productivity all showed higher values compared to cultivations where urea was employed. Furthermore, on medium containing urea, glucose consumption was significantly slower and the amount of glucose was not exhausted during cultivation. A possible reason for this is that both yeast extract and tryptone are complex supplements, which consist of nitrogen and additional organic compounds serving as carbon source, vitamins etc. The lower 2,3-BD production on medium containing urea may also be due to the incomplete utilization of glucose and the fact, that acetoin production started after 21 h as soon as maximum 2,3-BD concentration was reached. During the further cultivation time, the residual glucose was employed for acetion synthesis.

The cultivation experiments conducted in the attempt to reduce the amount of yeast extract and tryptone in the medium showed that the highest level of 2,3-BD (46.1 g/L) was obtained on medium with 5 g/L of each of the components. A simultaneous reduction in the amount of both complex components resulted in a corresponding lower production of 2,3-BD. However, by keeping the concentration of one of the compounds at the original level of 5 g/L and reducing the concentration of the other compound to 3.5 g/L (3/4 of the original concentration), the maximum level of 2,3-BD was 41.1 g/L (yeast extract reduction) and 41.7 g/L (tryptone reduction). These results are only 4.4-5 g/L lower than the best results obtained in this experimental series. This low concentration difference may be neglected, if the reduced input

of the complex compound, corresponding to a lower cost of the medium is more significant for an efficient industrial process than the slightly higher end-product concentration. A 1/4-reduction of the amount of yeast extractor tryptone is therefore possible, if the other component is added in the original concentration.

4.3.2 Shake flask experiments on wood hydrolysates

The comparison between 2,3-BD production by *B. licheniformis* DSM 8785 from natural and artificial wood hydrolysate with 30 g/L glucose revealed similar results for both cultivation experiments. Moreover, both yield and productivity values were higher for the cultivation on natural wood hydrolysate medium.

The inhibitory effects of furfural, 5-HMF, 5-hydroxybenzoic acid, vanillin, syringaldehyde, formic acid and acetic acid on glucose consumption and 2,3-BD production by *B. licheniformis* DSM 8785 were studied using different concentrations ranges. During cultivation, the potential inhibitory compounds are metabolized in different ways. The inhibition mechanism of the tested compounds has already been discussed in chapter 4.2.2.

According to the results obtained during cultivation in the presence of single inhibitory compounds, formic acid showed the strongest effect on microbial growth and 2,3-BD production; an inhibitory effect was observed from a 2-fold concentration compared to the original amount. On the other hand, vanillin shows the weakest inhibition among the tested compounds; an inhibitory effect on bacterial growth and 2,3-BD production could be detected from a 160-fold concentration. However, the relative amount of vanillin was generally significantly lower compared to the other compounds (see Tab. 3-20). 5-HMF and syringaaldehyde showed a medium effect, from concentration of 32-fold and 40-fold, respectively. The inhibitory influence of acetic acid started with the addition of a 4.25-fold concentration of the component. 4-Hydroxybenzoic acid and furfural inhibited bacterial growth and 2,3-BD production from 6-fold and 8-fold concentrations, respectively.

Regarding the experiments performed by adding a combination of potential inhibitory compounds, an effect already took place at the original concentration: during this cultivation, only approx. 15 g/L 2,3-BD were obtained, while in the absence of inhibitors an almost double amount of 2,3-BD was produced.

An inhibitory effect by organic acids could not be avoided by the adjustment of pH in the culture medium. The inhibitory effect observed in the presence of phenolic compounds appeared under relatively high compound concentrations. Furthermore, the inhibitory effect was enhanced by adding a combination of inhibitors, as compared to the original concentration of single components.

4.3.3 Scale-up to the 3.5 L bioreactor scale on different C-sources

The first experiment in the 3.5 L bioreactor scale tested three different stirring speeds between 200 and 500 rpm. A stirring speed of 400 rpm was found optimal for bacterial growth and 2,3-BD production. While low glucose consumption and OD_{540} values were observed at 200 rpm, higher stirring speeds (500 rpm) led to enhanced acetoin production. Regarding the optimization of the aeration rate, a value of 1.2 L/(L·min) was found to be optimal for 2,3-BD production in the 3.5 L bioreactor. With this aeration rate, a maximum 2,3-BD concentration as well as maximum values for yield and productivity could be detected.

Both the effect of the stirring speed and of the aeration rate on 2,3-BD production can be explained as follows: At a lower aeration, the oxygen supply is insufficient and the lower mixing leads to a lower interaction between cells and medium. On the contrary, a higher aeration rate causes a higher shear stress. Furthermore, high aeration rates show higher acetoin production at the expense of 2,3-BD synthesis. This is consistent with the findings of Laube et al. (1984a), who mentioned that high agitation values correspond to high aeration rates and lead to a decrease in 2,3-BD production.

Comparing the cultivation experiments performed in the 3.5 L bioreactor scale on artificial wood hydrolysate with / without addition of potential inhibitory compounds, the results were found to be very similar regarding maximum 2,3-BD production. The values obtained for both yield and productivity were somewhat lower in the presence of inhibitory compounds. These results contradict with the results obtained in the shake flask scale (see chapters 3.3.2 and 4.3.2), where an inhibiting effect was observed in the presence of a 1-fold concentration of inhibitors. A very probable explanation is that, in contrast to the shake flask experiments, for the cultivation in the 3.5 L bioreactor scale, the inhibitory compounds were added to the liquid medium and autoclaved at 121°C. Some compounds like formic acid may decompose during the autoclaving process and lose their inhibitory ability, as reported by Gale (2000).

The results of the cultivation in the 3.5 L bioreactor scale on natural wood hydrolysate medium were lower compared to the experiment on artificial wood hydrolysate medium. This is in accordance to the results obtained in the shake flask scale, which showed an inhibitory effect of the amount of compounds corresponding to a natural wood hydrolysate with 120 g/L glucose. However, the actual initial glucose concentration was only 100.6 g/L due to dilution by addition of liquid medium components and preculture. Furthermore, bacterial growth was slower on natural wood hydrolysate and a two-step preculture (first on glucose, then on natural wood hydrolysate) was required for microbial “adaptation” in contrast to shake flask scale experiments. Maximum 2,3-BD concentrations reached only 36 g/L on natural wood hydrolysate medium, while on artificial medium 47.6 g/L 2,3-BD were obtained. Apart from

inhibition effects and the lower initial glucose concentration in the medium, a reason for the lower product concentration might be the lower oxygen supply and transport of nutrients to the cells due to the slightly viscous natural wood hydrolysate medium. Moreover, the maximum 2,3-BD concentration was 10 g/L higher than for the corresponding cultivation in the shake flask scale (see chapter 3.3.2.2, Fig. 3-35; 1-fold concentration of combined inhibitors). However, the concentrations of inhibitors added to the medium were much higher for the shake flask experiments, based on earlier data from our project partners (see chapter 3.3.2.2 compared to chapter 3.3.3.4).

Three fed-batch cultivations were tested in the 3.5 L bioreactor scale and the best results were obtained when the cultivation was started with 120 g/L glucose and 135 g glucose (per 3 L culture) and corresponding amounts of the other nutrients were fed at a residual glucose concentration of 75 g/L. The feeding steps were carried out 5 times and the glucose level was increased by 45 g/L with each feeding. This strategy led to the highest 2,3-BD amount (127.4 g/L) as well as the highest yield and productivity.

Further information about the metabolism regarding the metabolic pathways employed by the bacteria can be gathered from the exhaust gas analysis. The most important role is played by the respiratory quotient (RQ). Zeng et al. (1994) reported that an RQ value of 4.0 is optimal for 2,3-BD production. RQ values higher than 4 are an indication of alcoholic fermentation and ethanol production, while values below 4 point to acetoin and acetate synthesis and an involvement of the citric acid cycle. In this experiment, after the initial part of the cultivation, RQ values oscillated around 4, increasing after each feeding, corresponding to the increased OUR.

The best results for the cultivation experiments carried out in the 3.5 L bioreactor scale using an increased initial glucose concentration of 180 g/L were obtained at an increased temperature of 37°C. The results showed that a higher incubation temperature is better for *B. licheniformis* DSM 8785 in order to adapt to higher initial substrate concentrations. The maximum 2,3-BD concentration reached 78.9 g/L; the yield was 0.46 g/g glucose, which is similar to the theoretical yield of 0.5 g/g glucose (Voloch et al. 1985) and the results obtained with risk class 2 bacteria (Ma et al. 2009; Ji et al 2010; Nie et al. 2011).

The results obtained at 30°C in the shake flask scale could not be reproduced in the 3.5 L bioreactor scale. After 40 h the cells reached a nearly stationary growth phase. In this growth phase, bacteria exhibit a reduced metabolism (Fuchs and Schlegel 2006) and therefore have lower oxygen requirements. Due to this fact, the importance of energy production via fermentation is reduced and NAD^+ regeneration is unnecessary. This would explain the low conversion of acetoin to 2,3-BD and consequent accumulation of acetoin in the culture broth.

4.3.4 Scale-up to the 45 L bioreactor scale on glucose

The results of the cultivation experiments performed using *B. licheniformis* DSM 8785 indicate that the scale-up of 2,3-BD production from the 3.5 L to the 45 L bioreactor scale is possible. The highest values for yield and productivity were reached, when the impeller tip speed was employed as scale-up criterion (170 rpm). However, the maximum 2,3-BD amount was slightly higher at a stirring speed of 160 rpm, corresponding to a constant aeration number as scale-up criterion; the yield was equal to the cultivation at 170 rpm. The corresponding cultivation experiments should however be repeated in order to ensure the reproducibility of the results.

Compared to the results obtained in the 3.5 L bioreactor scale, the experiments conducted in the 45 L bioreactor scale led to a lower maximum 2,3-BD amount and yield, while the productivity was higher than in the smaller bioreactor scale (see chapter 3.3.4.2, Tab. 3-30). Since the employed bioreactors showed no exact geometrical similarity (see chapter 3.3.4.1, Tab. 3-29) and in consequence possessed different hydrodynamics, the suitability of the impeller tip speed as scale-up criterion cannot be considered generally valid. Therefore suitable parameters have to be found for each bioreactor.

With increasing stirring speed, an increase in acetoin concentration and a decrease in glycerol production could be detected (see chapter 3.3.4.2, Tab. 3-30). This can be explained by the fact that the power and oxygen input are lower at low stirring speed values. This way, the bacteria have to produce the required energy by fermentation. When the oxygen supply is insufficient, the appropriate regeneration of co-factors like NAD^+ cannot be achieved by electron transfer onto oxygen. Therefore, other compounds (e.g. acetoin) must be reduced for co-factor regeneration. Another reaction leading to NAD^+ regeneration is the reduction of dihydroxyacetone phosphate to glycerol-3-phosphate, which is then dephosphorylated yielding glycerol (Spencer and Spencer 1978). This reaction represents an alternative to 2,3-BD production from pyruvate and is chosen by microorganisms under deficient oxygen supply conditions. Regarding acetoin production, the process is inverted: With increasing oxygen supply, a greater fraction of NADH is regenerated by the electron transport chain (with oxygen as electron acceptor) instead of by glycerol or 2,3-BD synthesis, because this way more energy in form of ATP can be generated. Consequently, acetoin accumulates, while 2,3-BD production decreases.

The impeller tip speed was proven to be a suitable scale-up criterion from the 3.5 L to the 45 L bioreactor scale also for the cultivation experiment conducted with an increased initial glucose concentration (180 g/L) and at an increased temperature (37°C). Furthermore, the almost identical time course for the 2,3-BD production in both bioreactor scales is remarkable (see chapter 3.3.4.3, Fig. 3-50). The productivities are consequently equal and the time course

for glucose consumption shows a parallel run for the two scales, the only difference being in the initial glucose concentration. This shows that the cultivation conditions were similar for the two bioreactor scales and the scale-up of 2,3-BD production was successfully implemented.

4.3.5 Conclusions on 2,3-BD production with *B. licheniformis* DSM 8785

Shake flask cultivations showed that a glucose concentration of 180 g/L, 30°C and 100 rpm are optimal conditions for high 2,3-BD production with *B. licheniformis* DSM 8785. 72.6 g/L 2,3-BD were obtained in batch experiments. Moreover, using the fed-batch cultivation mode, 2,3-BD production could be increased up to 144.7 g/L and the productivity up to 1.14 g/(L*h).

Cultivations on natural wood hydrolysates were similar to corresponding experiments on glucose medium or artificial wood hydrolysates. The amount of potential inhibitory compounds present in natural wood hydrolysates with 120 g/L glucose showed an inhibiting effect on bacterial growth and 2,3-BD production.

Regarding the 3.5 L bioreactor scale, 120 g/L glucose, 30°C, 400 rpm and an aeration rate of 1.2 L/(L*min) were detected as optimal conditions for a successful scale-up. During fed-batch cultivations, an increase in 2,3-BD up to 127.4 g/L could be detected. For higher glucose concentrations (180 g/L), a temperature of 37°C was better, leading to 78.9 g/L 2,3-BD.

A successful scale-up to the 45 L bioreactor scale was obtained using the impeller tip speed as scale-up criterion. From 154 g/L initial glucose, 65.1 g/L 2,3-BD could be reached.

The results obtained during these studies are very promising for large scale fermentation processes. 2,3-BD concentrations obtained in batch and fed-batch experiments are higher than any concentration reported so far from glucose using *B. licheniformis* (Nilegaonkar et al. 1992; Nilegaonkar et al. 1996; Perego et al. 2003; Wang et al. 2012) as well as other risk group 1 microorganisms (Biswas et al. 2012; Häßler et al. 2012; Ji et al. 2011; Yang et al. 2011). Furthermore, 2,3-BD concentrations reached in these studies are comparable to 2,3-BD production using risk group 2 strains. A summary of the highest 2,3-BD concentrations, yields and productivities reported until now in literature as well as the results of the present studies is given in Tab. 4-1. As a comparison, the maximum 2,3-BD concentration reported until now during fed-batch cultivation on glucose was 150 g/L, obtained using *Klebsiella pneumoniae* (Ma et al. 2009). The overall highest 2,3-BD production, 152 g/L, was reached from sucrose after fed-batch cultivation using *Serratia marcescens* (Zhang et al. 2010a, b). Therefore *B. licheniformis* DSM 8785 can be considered a very promising strain for 2,3-BD production in large scale fermentation processes.

Table 4-1 Comparison of the 2,3-BD production using different microorganisms

Microorganism	Substrate	Max. 2,3-BD [g/L]	Yield [g/g][*1]	Productivity [g/(L*h)]	Reference
Bacillus subtilis					
AJ1992	Glucose	2.5	0.38	0.33	Moes et al. (1985)
RB03	Glucose	6.1	0.33	0.41	Biswas et al. (2012)
Bacillus amyloliquefaciens					
B10-127	Glucose	92.3	0.15	0.96	Yang et al. (2011)
B10-127	Glucose	66.5	~0.42	2.22	Yang et al. (2011)
Bacillus licheniformis					
BL5	Glucose	12.2	0.45	2.65	Wang et al. (2012)
DSM 8785	Glucose	72.6[*2]	0.42	0.86	Shake flask scale (batch, 30°C)[*4]
DSM 8785	Glucose	144.7	0.40	1.14	Shake flask scale (fed-batch, 30°C)[*4]
DSM 8785	Glucose	78.9	0.46	1.18	3.5 L BR scale (batch, 37°C)[*4]
DSM 8785	Glucose	127.4	0.37	1.05	3.5 L BR scale (fed-batch, 30°C)[*4]
DSM 8785	Glucose	65.1	0.42	1.19	45 L BR scale (batch, 30°C)[*4]
Paenibacillus polymyxa					
DSM 365	Glucose	111.0[*3]	-	2.06	Häßler et al. (2012)
Enterobacter aerogenes					
DSM 30053	Glucose	110.0	0.49	5.40	Zeng et al. (1991)
EMY-01	Glucose	118.0	-	2.19	Jung et al. (2012)
Klebsiella pneumonia					
SDM	Glucose	150.0	0.43	4.21	Ma et al. (2009)
Klebsiella oxytoca					
ME-UD-3	Glucose	130.0	0.48	1.64	Ji et al. (2010)
CCTCCM207023	Glucose	127.9	0.48	1.78	Nie et al. (2011)
Serratia marcescens					
H30	Sucrose	152.0	0.41	2.67	Zhang et al. (2010a)
H30	Sucrose	139.9	0.47	3.49	Zhang et al. (2010b)

[*1] 2,3-BD yield is given in g/g substrate (glucose, sucrose); the sugars contained in complex nutrients (e.g. yeast extract) are not considered

[*2] additional 10 g/L yeast extract and 10 g/L tryptone were used for cultivation

[*3] additional 60 g/L yeast extract were used for cultivation

[*4] results obtained in the present thesis

5 Summary

The objective of the present PhD thesis was the development of an efficient fermentative process for 2,3-butanediol production from wood hydrolysates using non-pathogenic microorganisms, comparable to risk group 2 strains.

The first task was the screening for suitable non-pathogenic microorganisms, including strains from culture collections, project partners as well as isolated microorganisms from environmental habitats and their potential for 2,3-BD production was investigated. A strain improvement by random mutagenesis using UV light was attempted for the best strains, however without success. The best strains, *P. polymyxa* ATCC 12321 and *B. licheniformis* DSM 8785, were subjected to several optimizations in the shake flask and bioreactor scale.

In the shake flask scale initial optimizations regarding medium composition (carbon and nitrogen source and concentration) and cultivation parameters (temperature, shaking rate) were carried out during batch cultivations with free and immobilized cells. From 68 g/L glucose at 25°C and 80 rpm, 24.4 g/L 2,3-BD were reached with *P. polymyxa*. With *B. licheniformis* 72.6 g/L 2,3-BD were obtained from 180 g/L glucose at 30°C and 100 rpm.
Using the fed-batch cultivation mode and a stepwise addition of nutrients, 2,3-BD production could be increased up to 63.3 g/L (*P. polymyxa*) and 144.7 g/L (*B. licheniformis*).
Cultivation results were similar on glucose medium, natural and artificial wood hydrolysates. The amount of potential inhibitors present in natural wood hydrolysates with 68 g/L glucose showed no negative effect on cultivations with *P. polymyxa*, while *B. licheniformis* was inhibited by compound concentrations corresponding to a hydrolysate with 120 g/L glucose.

With optimized parameters, a scale-up of 2,3-BD production to the 3.5 L bioreactor scale was carried out. 30°C, 400 rpm and an aeration rate of 1.2 L/(L*min) were detected as optimal conditions for a successful scale-up. For *P. polymyxa*, an increase in productivity could be obtained using forced pH fluctuations from 5.3 → 5.8. A temperature of 37°C was better for *B. licheniformis* at higher glucose concentrations (180 g/L), leading to 78.9 g/L 2,3-BD. During fed-batch cultivations, up to 127.4 g/L 2,3-BD could be reached using *B. licheniformis*.

A successful scale-up to the 45 L bioreactor scale was obtained using the impeller tip speed as scale-up criterion. From 120 g/L glucose, 42 g/L 2,3-BD were reached, while 65.1 g/L 2,3-BD were obtained using a higher amount of initial glucose (154 g/L).

The results indicate the highest 2,3-BD concentrations reported so far using GRAS microorganisms and lie in the same range with data described for risk group 2 strains. *B. licheniformis* DSM 8785 is a suitable candidate for large scale 2,3-BD production processes.

6 Zusammenfassung

Das Ziel dieser Arbeit war die Entwicklung eines effizienten fermentativen Prozesses für die 2,3-Butandiolbildung aus Holzhydrolysaten. Dabei sollten nicht-pathogene Mikroorganismen verwendet werden und vergleichbare Ergebnisse zu Risikoklasse 2 Stämmen erzielt werden.

Die erste Aufgabe war das Screening nach geeigneten nicht-pathogenen Mikroorganismen aus Stammsammlungen, von Projektpartnern sowie aus Umweltproben isoliert und deren Untersuchung hinsichtlich 2,3-BD Bildung. Eine Optimierung mittels UV Bestrahlung wurde für die besten Stämme erforscht, leider ohne Erfolg. Die besten Stämme, *P. polymyxa* ATCC 12321 und *B. licheniformis* DSM 8785, wurden mehreren Optimierungsschritten im Schüttelkolben- und Bioreaktormaßstab unterzogen.

Im Schüttelkolbenmaßstab wurden Optimierungen hinsichtlich der Medienzusammensetzung (C-/N-Quelle und Konzentration) und Kultivierungsbedingungen (Schüttelgeschwindigkeit, Temperatur) während Batch-Kultivierungen mit freien/immobilisierten Zellen durchgeführt. Von 68 g/L Glucose wurden mit *P. polymyxa* bei 25°C und 80 rpm 24,4 g/L 2,3-BD erzielt. *B. licheniformis* konnte 72,6 g/L 2,3-BD aus 180 g/L Glucose bei 30°C und 100 rpm bilden.
Während Fed-Batch-Kultivierungen mit schrittweise Zugabe von Nutrienten konnte die 2,3-BD Produktion auf 63.3 g/L (*P. polymyxa*) und 144.7 g/L (*B. licheniformis*) erhöht werden.
Kultivierungen auf Glucose bzw. natürlichem/künstlichem Holzhydrolysat wiesen ähnliche Ergebnisse auf. Die Hemmstoffmengen in einem Holzhydrolysat mit 68 g/L Glucose zeigten keine Inhibierung von *P. polymyxa* an. *B. licheniformis* wurde aber durch die Konzentrationen an Hemmstoffen inhibiert, die einem Holzhydrolysat mit 120 g/L Glucose entsprechen.

Mit optimieren Parametern wurde ein Scale-up der 2,3-BD Produktion zum 3,5 L Bioreaktormaßstab durchgeführt. 30°C, 400 rpm und eine Belüftungsrate von 1,2 L/(L*min) erwiesen sich als optimal für ein erfolgreiches Scale-up. Durch den Einsatz erzwungener pH-Shifts von 5,3 → 5,8 konnte ein Anstieg der Produktivität für *P. polymyxa* erreicht werden. Bei hohen Konzentrationen an Glucose (180 g/L) zeigten sich 37°C besser geeignet für *B. licheniformis*. Während Fed-Batch-Kultivierungen mit *B. licheniformis* wurden 127.4 g/L 2,3-BD gemessen.

Unter Verwendung der Rührerspitzengeschwindigkeit als Scale-up Kriterium konnte ein gelungenes Scale-up zum 45 L Bioreaktormaßstab durchgeführt werden. 120 g/L Glucose führten zu 42 g/L 2,3-BD; aus 154 g/L Glucose konnten 65,1 g/L 2,3-BD erzielt werden.

Die Ergebnisse weisen die höchsten 2,3-BD Konzentrationen auf, die bisher mit GRAS Mikroorganismen erzielt wurden und in der Größenordnung der Risikoklasse 2 Stämme liegen. *B. licheniformis* DSM 8785 ist für die 2,3-BD Produktion im großen Maßstab geeignet.

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8 Abbreviations

2,3-BD	2,3-Butanediol
5-HMF	5-Hydroxymethylfurfural
ATCC	American Type Culture Collection
ATP	Adenosine-5'-triphosphate
CCD	Central Composite Design
CDW	Cell dry weight
CECT	Colección Española de Cultivos Tipo (Spanish Type Culture Collection)
CER	Carbon dioxide evolution rate
DNS	3,5-Dinitrosalicylic acid
DoE	Design of Experiments
DSMZ	Deutsche Sammlung für Mikroorganismen und Zellkulturen (German Collection of Microorganisms and Cell Cultures)
ERA-IB	European Research Area Network for Industrial Biotechnology
GRAS	Generally Regarded As Safe
HPLC	High performance liquid chromatography
IUPAC	International Union of Pure and Applied Chemistry
M	Molar mass [g/mol]
NAD^+/NADH	Nicotinamide adenine dinucleotide
NB	Nutrient broth
N_B	Aeration number
NRRL	Northern Regional Research Laboratory
n.s.	Not specified
OD	Optical density
OUR	Oxygen uptake rate
P	Volumetric productivity [g/(L·h)]
p^N	Pressure under standard conditions
pO_2	Oxygen partial pressure
PUBB	Production and Upgrading of 2,3-Butanediol from Biomass
R	Universal gas constant
rDNA	Ribosomal desoxyribonucleic acid
Re	Reynolds number
RI	Refractive index
RQ	Respiratory quotient
T^N	Temperature under standard condition (273.15 K)
TCA cycle	Tricarboxylic acid cycle
TLC	Thin layer chromatography
V'^N	Volume stream under standard condition
v_s	Superficial gas velocity
v_{tip}	Impeller tip speed
$Y_{P/S}$	Yield coefficient [g substrate/g product]
χ^A	Mole fraction of oxygen or carbon dioxide in the exhaust air (outlet)
χ^E	Mole fraction of oxygen or carbon dioxide in the inlet air

9 Appendix

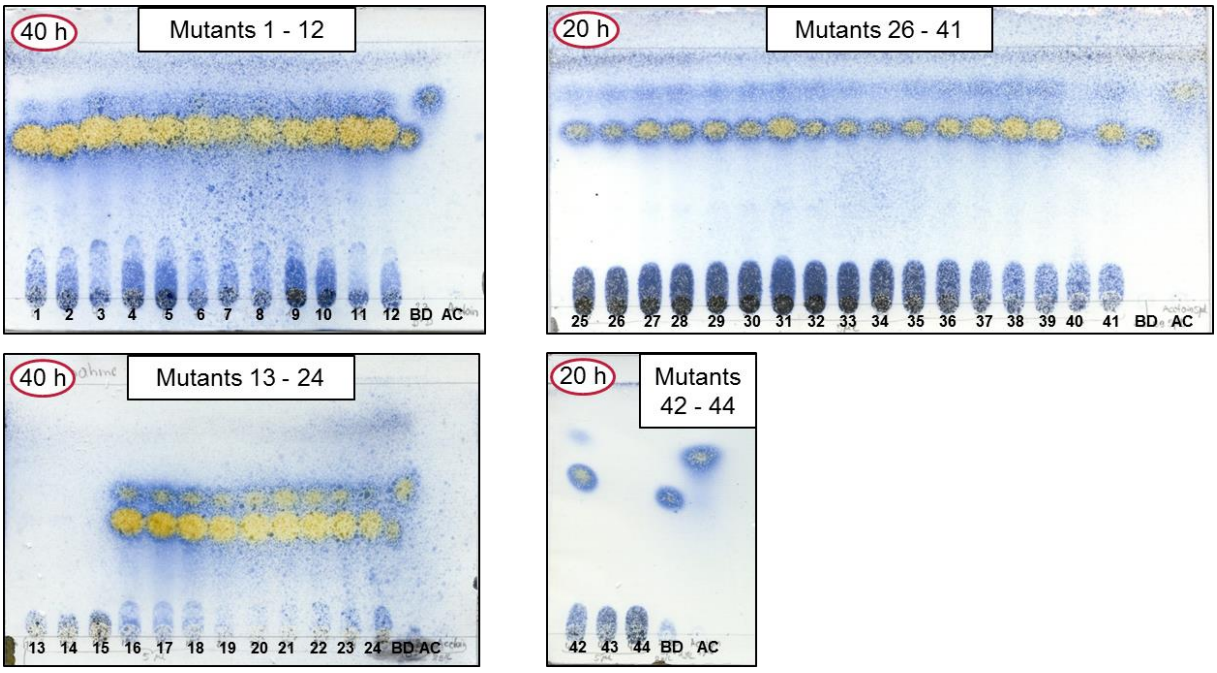

Figure 9-1 TLC results from 96-well microplate cultivation of potential UV-mutants from *P. polymyxa* ATCC 12321; 5 µL samples (incl. 2,3-BD and Acetoin standards) [joint work with Iphöfer, 2011]

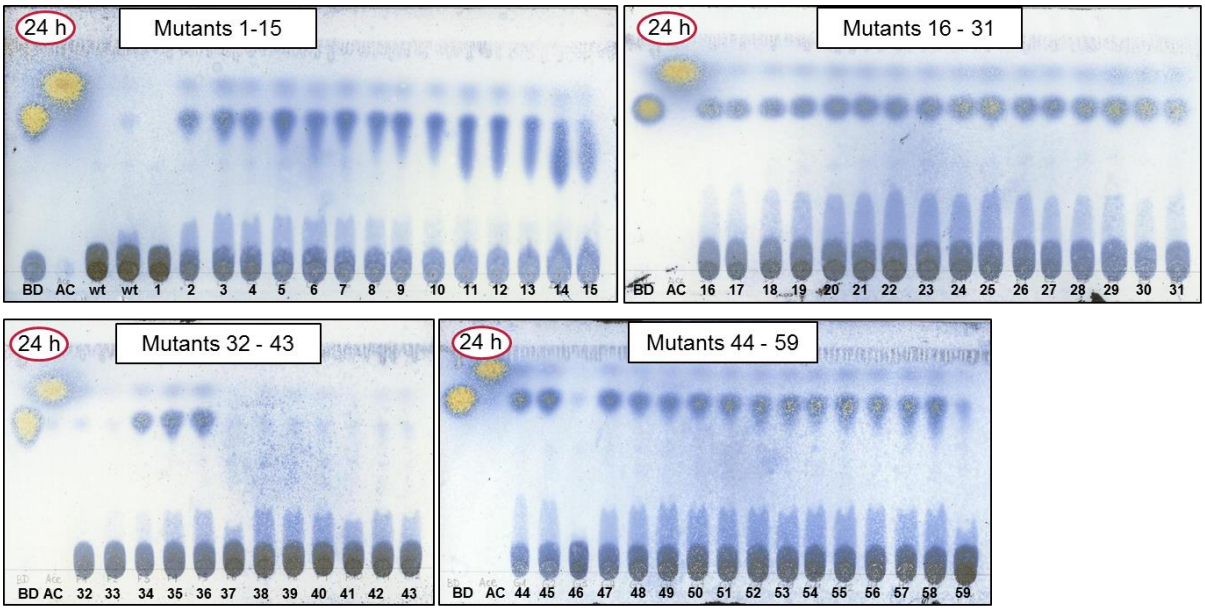

Figure 9-2 TLC results from 96-well microplate cultivation of potential UV-mutants from *B. licheniformis* DSM 8785; 5 µL samples (incl. 2,3-BD and Acetoin standards) [joint work with Zhou, 2012]

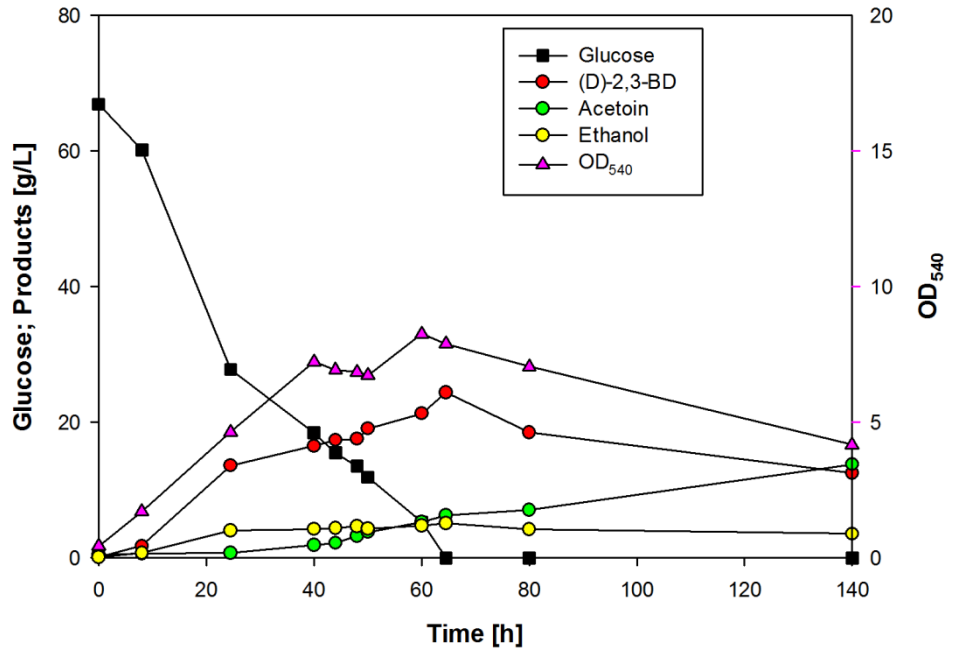

Figure 9-3 Time course for the cultivation of *P. polymyxa* ATCC 12321. Conditions: 500 mL shake flasks, 100 mL medium (68 g/L glucose), 25 °C, 80 rpm, initial pH 6.6 (not adjusted) [joint work with Iphöfer, 2011]

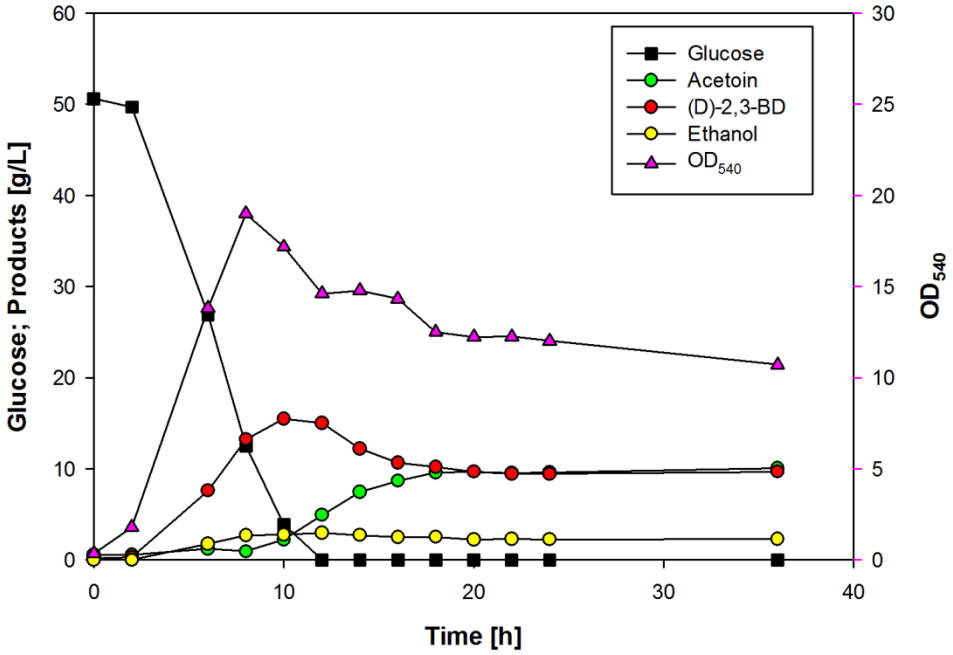

Figure 9-4 Time course for the cultivation of *P. polymyxa* ATCC 12321. Conditions: 500 mL shake flasks, 100 mL medium (50 g/L glucose), 35 °C, 150 rpm, initial pH 6.6 (not adjusted) [joint work with Iphöfer, 2011]